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Hemoproteins of Enterococcus faecalis

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Department of Cell and Organism Biology, Lund University

Hemoproteins of
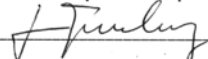# *Enterococcus faecalis*

Lena Frankenberg

Akademisk avhandling

som för avläggande av filosofie doktorsexamen vid Naturvetenskapliga fakulteten vid Lunds Universitet kommer att försvaras i Biologihörsalen, Biologihuset, Sölvegatan 35, fredagen den 7 mars 2003, kl. 10.00.
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Abstract

The gram-positive bacterium *Enterococcus faecalis* is a commensal organism, often found in the gut of humans and other animals. It can cause opportunistic disease and it frequently causes nosocomial infections. Treatment of such infections is often impaired by this organisms broad intrinsic and acquired resistance to antibiotics. *E. faecalis* uses homo-lactic fermentation for energy production. Work done in the 1970'ies indicated that production of cytochromes and respiratory activity occur if the cells are supplied with heme. A decade later, heme-dependent catalase activity was reported.

This thesis describes the identification of a cytochrome *bd* and a catalase in *E. faecalis*. These two enzymes are synthesized only if heme is supplied in the growth medium. The cytochrome *bd* most likely functions as terminal oxidase in an aerobic respiratory chain present in *E. faecalis* under some conditions. The *E. faecalis* catalase was overproduced, purified and its major biochemical characteristics were determined. In addition, a catalase-deficient mutant was constructed.

The newly acquired knowledge about the *E. faecalis* catalase was used to establish an in vivo system for production of artificial hemoproteins. It was demonstrated that *E. faecalis* cells can take up a variety of heme analogues from the growth medium and integrate them into the cytoplasmic catalase protein.

Key words: Enterococci, heme, catalase, cytochrome, porphyrin

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Department of Cell and Organism Biology, Lund University

Hemoproteins of
Enterococcus faecalis

Lena Frankenberg

PhD thesis in Microbiology

2003

Cover picture:
Crystals of *E. faecalis* catalase. Crystallization was performed in 50 mM Bis(2-hydroxyethyl)-imino-tris(hydroxymethyl)methane pH 7.0, 1.6 M $LiSO_4$. Crystallization and photography were done by Kjell Ove Håkansson, August Krogh Institute, University of Copenhagen.

Published papers and manuscripts

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I   Winstedt L, L. Frankenberg, L. Hederstedt and C. von Wachenfeldt. (1999) ***Enterococcus faecalis* V583 contains a cytochrome *bd*-type respiratory oxidase.** J. Bacteriol. 182:3863-3866.

II   Frankenberg, L. M. Brugna and L. Hederstedt. (2002) ***Enterococcus faecalis* heme-dependent catalase.** J Bacteriol. 184:6351-6356.

III   Brugna, M. L. Frankenberg and L. Hederstedt. (2003**)** *In vivo* **synthesis of artificial catalase proteins containing heme analogues.** *Manuscript.*

IV   Frankenberg, L. M. Brugna and L. Hederstedt. (2003) **Construction and analysis of an *E. faecalis* catalase-deficient mutant.** *Manuscript.*

Contents

1 Introduction

1.1 General background

Heme belongs to a family of compounds named porphyrins. Porphyrins are found as prosthetic groups in biologically very important proteins. These proteins are involved in fundamental cellular processes in plants, animals and most bacteria.

A porphyrin is composed of a ring of four pyrrole residues carrying various side chains and connected by methine bridges. Several of the most interesting chemical properties of porphyrins are due to the conjugated bond system of the porphyrin macrocycle. An easily observed consequence of the conjugated bond system is that porphyrins appear intensely colored, due to the absorption of visible light.

Heme is a planar porphyrin with a chelated iron atom in the center of the molecule (Figure 1). Heme has unique redox properties useful for example in electron transport reactions and chemical reactions involving oxygen. Accordingly, heme is found in proteins like cytochromes, catalase and hemoglobin.
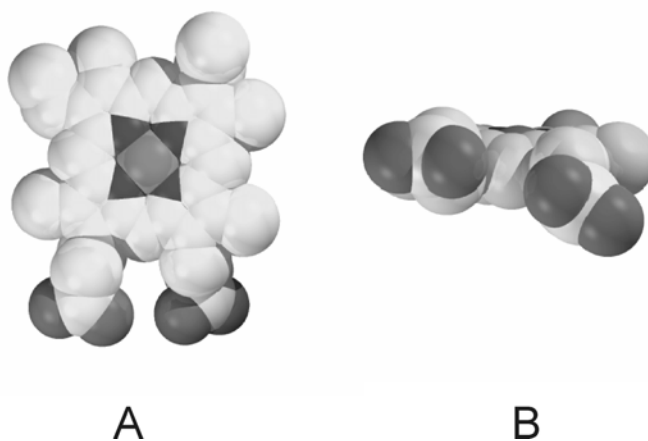
Figure 1. Space-filling model of heme B (protoporphyrin IX).

Studies of heme metabolism at the cellular level are problematic since heme is an essential molecule to most of the commonly used model organisms. Disturbances of heme metabolism are prone to have drastic effects on the cell. In the present study, this problem is circumvented by exploiting the unique features of *Enterococcus faecalis* heme metabolism. This bacterium does not require heme for growth, but if supplied with heme, it can synthesize hemoproteins (Knowles, 1980).

1.2 Heme

The nomenclature of hemes and porphyrins appears rather complicated. Several trivial names and the use of a series of different systematic nomenclature systems have created today's situation where, in some cases, several names designate the same compound. Thus, **Fe(II) protoporphyrin IX,** suggested by Hans Fisher (1881-1945), is also called **protoheme IX** and **heme B**. **Hemin** is the chloride salt of heme B. The Fisher nomenclature is still widely used, at least for the most common tetrapyrroles. Nevertheless, the IUPAC (International Union of Pure and Applied Chemistry) nomenclature committee has established a new nomenclature for the tetrapyrroles. For heme B, they propose the rarely used name **proto-porphyrinato iron(II)** or the systematic **8,13-divinyl-3,7,12,17-tetramethyl-porphyrin-2,18-dipropionato iron(II).**

Several types of hemes can be found in biological molecules, for example heme A, heme C and heme D. The biosynthetic precursor of them all is heme B (Figure 2). Heme B carries propionyl groups at positions 6 and 7 of the porphyrin ring (numbering according to the Fisher system), and vinyl groups at positions 2 and 4. Four methyl groups occupy the remaining β-carbons (denomination in the Fischer nomenclature system of the pyrrole carbon atoms which can carry a substituent). The nitrogen atoms in the center of the molecule together coordinate the ferrous iron.

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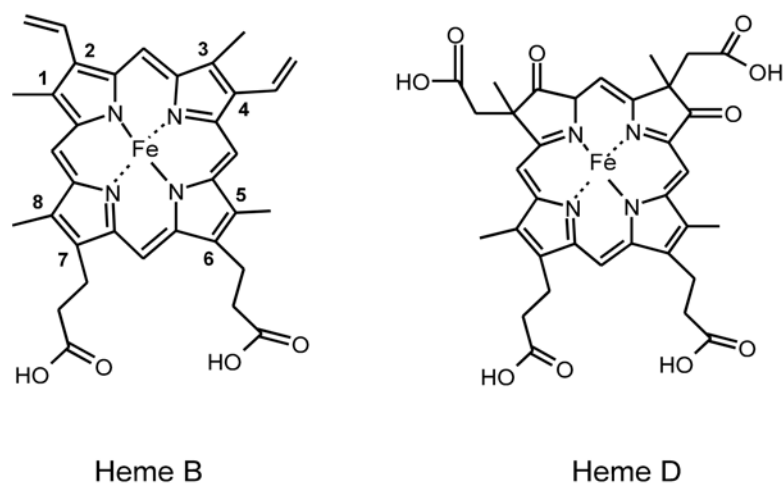
Figure 2. Model of heme B and heme D (Murshudov *et al.*, 1996). The β-carbons are numbered according to the Fisher nomenclature.

Ferrous ion (Fe^{2+}) is highly reactive and therefore a very useful cofactor in biological systems. However, its high reactivity can be dangerous unless tightly controlled. In the presence of oxygen, reactions of the ferrous ion can lead to production of toxic hydroxyl radicals ($HO^{·}$), thereby initiating cascades of free radical reactions. These radicals can ultimately cause random oxidation of cell constituents such as membrane lipids and DNA (Borg and Schaich, 1987). The ferrous ion is also reactive when it is chelated by protoporphyrin. In biological systems, it is therefore important that both ferrous ions and heme is securely maintained in e.g. proteins, making access of molecules to the ion restricted. This is well illustrated by the strategy adopted by the *Plasmodium* parasite (the causative agent of malaria), which risks facing toxic levels of free heme accumulating due to the degradation of host hemoglobin by the parasite. The parasite has solved this problem by a detoxification mechanism involving the formation of a crystalline heme compound, hemozoin, where the polymerization of heme reduces its reactivity (for a recent review, see Ziegler *et al*., 2001). Classical anti-malarial drugs, such as quinine, inhibit the formation of hemozoin.

The presence of polar groups at one end and nonpolar groups at the other end of heme confers a strong amphiphilic character to the molecule. Due to the rather large size and the charge of the heme molecule, the rate of diffusion of heme across biological membranes is probably too low to be of physiological relevance. Heme is poorly soluble in water at physiological pH, but dissolves in alkaline solution due to the deprotonation of the propionic acid groups. In aqueous solution, heme has a strong tendency to aggregate as micelles, due to its amphiphilic character, and forms oxo-dimers in the presence of oxygen (Figure 3; Kuzelova *et al.*, 1997). Heme oxy-dimers are thought to be less accessible to heme-binding proteins, and do not retain the catalytic activity of monomeric heme.
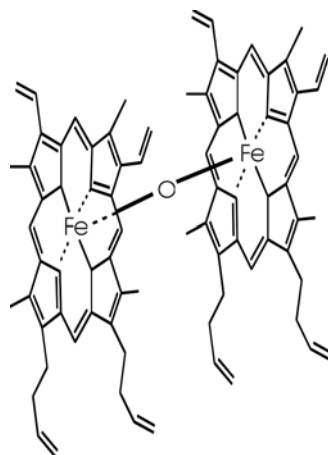
Figure 3. Schematic representation of a heme oxo-dimer.

E. faecalis is thus confronted with four major problems concerning heme utilization: Heme is poorly soluble, highly reactive, diffuses very slowly across membranes and has a tendency to form oxy-dimers. Therefore, it is highly probable that *E. faecalis* and other cells contain specific intracellular heme carriers, which are active in uptake, transport and insertion of heme into hemoproteins. The carriers are thought to facilitate heme acquisition, prevent aggregation and hinder toxic effects on cell constituents. Except for a set of heme uptake proteins described in bacteria and involved in iron metabolism, no such cellular carriers have yet been identified.

1.3 The bacterium *E. faecalis*

E. faecalis is a gram-positive bacterium formerly known as *Streptococcus faecalis*. It is a common inhabitant of the intestine of animals including humans, where it is part of the normal, commensal flora (Devriese *et al.*, 1991). However, *E. faecalis* can cause disease in immuno-deficient persons and it is frequently the causative agent of nosocomial (hospital-acquired) infections. The emergence of multi-drug resistant strains has become a serious problem (Huycke *et al.*, 1998). Research performed by the pharmaceutical industry and independent researchers around the world is aimed at expanding the knowledge about the genus *Enterococcus*, partly in order to combat the hospital-acquired infections more efficiently. In line with these efforts, the Institute of Genomic Research (TIGR) has determined the whole genome sequence of *E. faecalis* strain V583. A prerelease of the sequence is available at the TIGR website (http://www.tigr.org). Strain V583 was chosen for the sequencing project because it is a clinical isolate resistant to high-levels of vancomycin, the last-resort antibiotic for drug resistant enterococci. Other *E. faecalis* strains, such as the plasmid-free strain OG1RF (Wirth *et al.*, 1986), have been kept as laboratory strains for decades and are better characterized than strain V583 (Sahm *et al.*, 1989).

E. faecalis is easily cultured in the laboratory, and pathogenicity is not of great concern under normal laboratory conditions. Some genetic tools, such as transformation procedures and cloning vectors, are available for enterococci (Manganelli *et al.*, 1998; Shepard and Gilmore, 1995; Wirth *et al.*, 1986). This, and the available genome sequence, was among our motives for choosing *E. faecalis* as our model organism. The main reason, however, was reports of facultative production of hemoproteins in *E. faecalis*. Most hemoprotein-producing organisms, to this date characterized, are unable to maintain life without heme. Known exceptions are some *Enterococcus* species, and closely related *Lactococcus* species (Sijpesteijn, 1970).

The physiology of *E. faecalis* is commonly described as facultatively anaerobic and the energy metabolism as homo-lactic fermentative. However, experimental work done in the 1960'ies and 1970'ies indicated that *E. faecalis* cells are capable of aerobic respiration if supplied with heme in the growth medium (Bryan-Jones and Whittenbury, 1969; Pritchard and Wimpenny, 1978; Ritchey and

Seeley, 1974). The presence of unspecified cytochromes and catalase activity was reported. We have confirmed these observations and characterized an enterococcal cytochrome *bd* (Paper I) and a heme-containing catalase (Paper II) in *E. faecalis*. After demonstrating the presence of these hemoproteins, we exploited *E. faecalis* for synthesis of artificial catalases (Paper III) and constructed a catalase-deficient *E. faecalis* mutant strain (Paper IV).
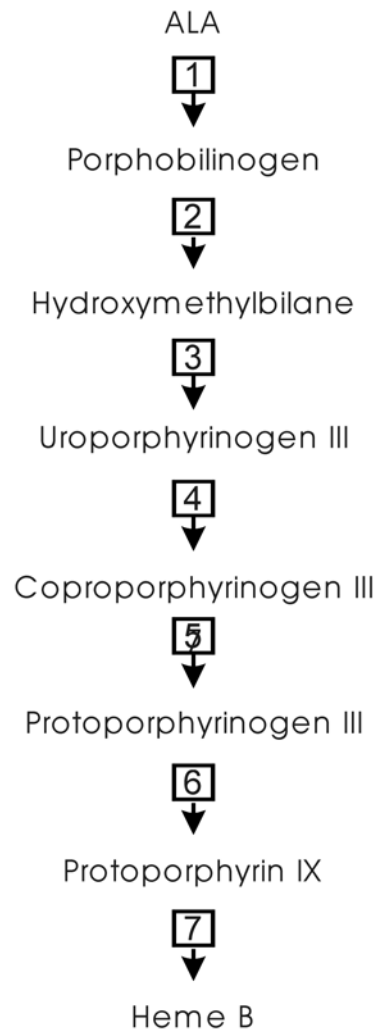
Figure 4. Heme B biosynthesis. The biosynthetic enzymes, symbolized by boxes, are numbered as follows: 1: ALA dehydratase, 2: Porphobilinogen deaminase, 3: Uro III synthase, 4: Uro III decarboxylase, 5: Coprophorphyrin III oxidase, 6: Protoporphyrinogen IX oxidase, 7: Ferrochelatase.

2 Heme metabolism

## 2.1 Heme biosynthesis and synthesis of hemoproteins

Most heme-requiring organisms can synthesize their own heme. In all these organisms, the synthesis of heme starts from the same compound, 5-aminolevulinic acid (ALA). In plants, Archaea and in most bacteria, ALA is synthesized from glutamyl-tRNA. In animals, fungi and α-proteobacteria, however, ALA is synthesized from succinyl-CoA and glycine. In bacteria, all steps in heme synthesis take place in the cytoplasm. The heme biosynthetic pathway of the gram-positive model organism *Bacillus subtilis* is shown in Figure 4.

The assembly of hemoproteins is not as well characterized as the heme biosynthesis. Many genes encoding the structural subunits of hemoproteins have been identified, in a multitude of different organisms. In all cases studied, the insertion of the heme group takes place after the synthesis of the apo-protein has been completed. However, it is not known in any organism how the heme is transported to the apoprotein, neither by which mechanism the actual insertion takes place.

In the case of cytochrome *c*, some experimental data are available regarding its biosynthesis and a model of hemoprotein assembly has been formulated (Thöny-Meyer, 1997). *C*-type cytochromes have heme covalently attached via thioether bonds formed between two cystein-residues of the polypeptide and the vinyl groups of the heme. Cytochrome *c* is located on the outside of the cytoplasmic membrane in prokaryotes. According to the model for cytochrome *c* biogenesis in bacteria (Figure 5), apo-cytochrome *c* is synthesized in the cytoplasm and then translocated across the cell membrane and folded on the outside. At this location, heme is inserted and covalently attached to the protein. Exactly how heme is transported across the membrane and incorporated into the apo-cytochrome is not known, but it involves transient covalent attachment of heme to the CcmE protein (Schulz *et al*., 1998).
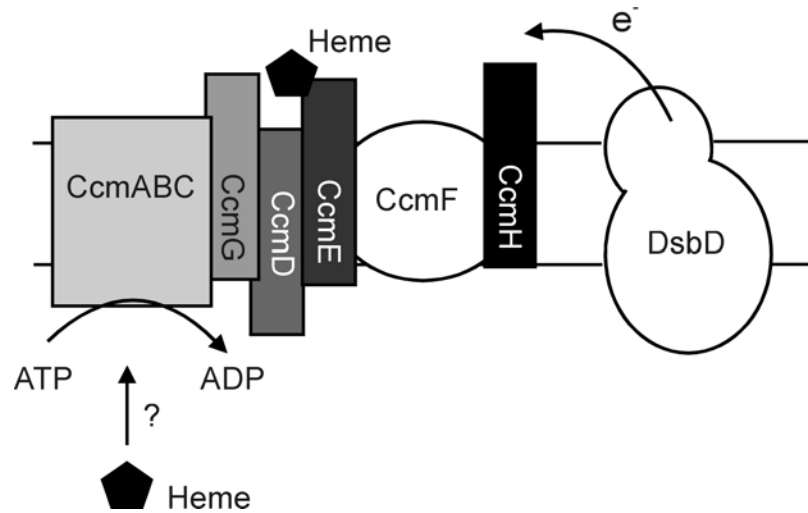
Figure 5. Overview of cytochrome *c* biogenesis in *Escherichia coli*. The apo-protein is exported to the periplasm by the Sec-machinery (not shown), while heme is transported by a separate route. The heme molecule is thought to be transported across the membrane by an ABC-transporter, CcmABC. Once in the periplasmic space, heme is covalently, but transiently, attached to the heme chaperone CcmE by an unknown mechanism. The proposed heme lyase CcmFH is involved in the final, covalent attachment of heme to apo-cytochrome *c*. Electrons, needed for the reduction of the cytochrome *c* cysteine residues, come via the DsbD protein.

2.2   Heme in *E. faecalis*

E. faecalis does not require heme for growth, but the growth yield is slightly promoted if hemin is added to the medium (Figure 6; Bryan-Jones and Whittenbury, 1969). This effect is observed with hemin concentrations ranging from 2 mg/l to 20 mg/l (own unpublished data).
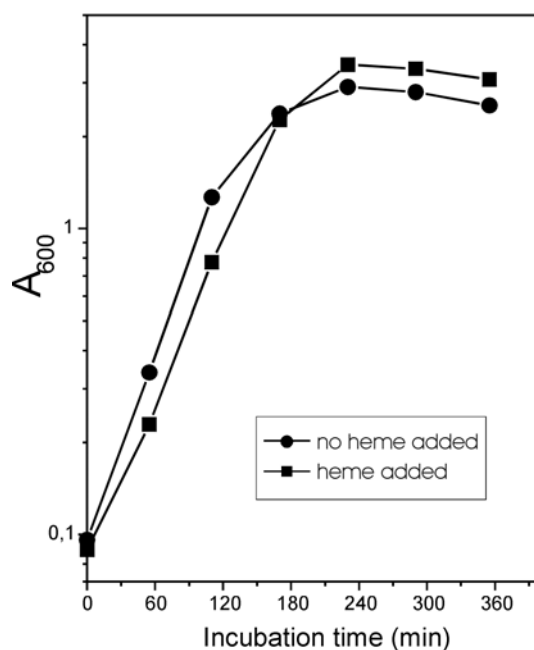
Figure 6. Representative growth curves of *E. faecalis* V583 at 37°C under oxic conditions, in a growth medium containing 10 g/l tryptone, 5 g/l yeast extract, 4 g/l glucose and 3 g/l K$_2$HPO$_4$ with 20 mg/l hemin added as indicated.

E. faecalis is unable to synthesize heme. Enzymes necessary for the conversion of ALA to protoporphyrin IX seem to be absent in *E. faecalis* as judged from the available genome sequence data. However, there is a gene apparently encoding a ferrochelatase (HemH), catalyzing the insertion of a ferrous ion into protoporphyrin IX. The presence of a HemH homologue in a bacterium that lacks the

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porphyrin biosynthetic pathway is enigmatic. Ferrochelatase has been suggested to catalyze not only the insertion of a ferrous ion into the porphyrin ring, but also the removal of this ion under certain circumstances (Loeb, 1995). If so, ferrochelatase would allow the use of heme as an iron source. This hypothesis has been much contested, because the removal of the ferrous ion from an intact porphyrin ring is highly unfavorable for thermodynamic reasons (Sigfridsson, 2001). A more plausible explanation for the presence of a ferrochelatase in *E. faecalis* is that this would allow the cell to use not only heme but also protoporphyrin IX for the synthesis of hemoproteins. In line with these thoughts, experiments have been performed in which hemin or heme-containing compounds in the growth medium have been substituted with protoporphyrin IX (Bryan-Jones and Whittenbury, 1969; section 6.4). Bryan-Jones and Whittenbury were unable to detect hemoproteins under these conditions while our results (Paper III) show that catalase is produced but contains protoporphyrin IX instead of heme. Thus, the role of HemH in *E. faecalis* remains unknown.

It is worth noticing that a gene named *hemK* is present in the *E. faecalis* annotated sequence. HemK is however a methyltransferase (Heurgue-Hamard *et al*., 2002), not involved in heme biosynthesis.

2.3   Bacterial heme acquisition

Iron is often a limiting factor for bacterial growth in natural environments. This is remarkable since iron is the second most abundant metal in the earths crust. However, as an ion it is usually present in the insoluble ferric (Fe^{3+}) form, and not in the more easily assimilated ferrous form. Consequently, bacteria need to have efficient uptake and storage mechanisms for iron, as well as recycling of iron from their own degraded proteins. This is true also for mammals, which even use iron sequestering as an anti-bacterial strategy: all iron in the body is tightly bound to different proteins, e.g. transferrin and lactoferrin, during transport and storage. Therefore, most pathogenic bacteria must be able to assimilate host iron present in proteins and other compounds. An important iron source for these bacteria is heme and abundant heme-containing proteins, e.g. hemoglobin, myoglobin and haptoglobin (Braun and Killman, 1999).

Bacteria have developed different strategies for the utilization of heme present in their environment (Goldman *et al*., 1998; Wandersman and Stojiljkovic, 2000). Commonly, an assembly of proteins acts together in an acquisition system, active in heme binding, transport and degradation. These uptake systems have primarily been characterized as systems that allow the use of heme as an iron source. Many of them have later been shown to enable the bacteria to use exogenous heme as a porphyrin source (Lewis *et al*., 1998; Mills and Payne, 1995; Turner *et al*., 1998; Worst *et al*., 1999). This indicates that in these systems, heme is transported as an intact molecule across the membrane.

The HmuTUV system will be discussed in section 3.3.2.

2.4   Heme degradation

Heme degradation has been studied mainly in mammals, where it is important in the turnover of red blood cells. Defective erythrocytes are destroyed, mainly in the spleen and in the liver, thereby releasing hemoglobin. Proteases degrade the globin part of the protein, while an enzyme, heme oxygenase, catalyzes the first step in the degradation of heme: the oxidative opening of the porphyrin ring. The opening of the ring destabilizes the molecule and the iron atom can be extracted and recycled. Mainly mammalian heme oxygenases have been characterized but a few bacterial heme oxygenases that use exogenous heme as an iron source have also been studied.

The enzymatic mechanism of human heme oxygenase (HO-1) is well known; see Montellano, 2000, for a review on this subject. HO-1 uses electrons from NADPH-cytochrome P-450 reductase and molecular oxygen in the oxidative degradation of heme. The end products are biliverdin, CO and iron. The reaction is initiated when an electron donated from the reductase reduces protoporphyrin iron (III) to protoporphyrin iron (II). Binding of oxygen to the reduced iron followed by a second electron transfer, results in a ferric peroxide complex. This complex self-hydroxylates the porphyrin ring, integrating a hydroxyl group at the α-meso-position. In the following catalytic step, an oxygen atom replaces the meso-carbon. The elimination of the meso-carbon and its hydroxyl group yields CO and verdoheme. Further oxidation integrates another oxygen atom at the meso-position, resulting in the opening of the porphyrin ring and release of iron and the waste product biliverdin. Biliverdin is

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ultimately converted to the bile pigment bilirubin diglucuronide and secreted into the small intestine.

The bacterial heme oxygenase, HmuO, is less well characterized, but it has been shown to catalyze a similar reaction (Figure 7; Wilks and Schmitt, 1998; Zhu *et al*., 2000). HmuO has extensive sequence similarity with the human HO-1 protein and the synthesis of both HO-1 and HmuO is repressed by iron and induced by heme (Schmitt, 1997).
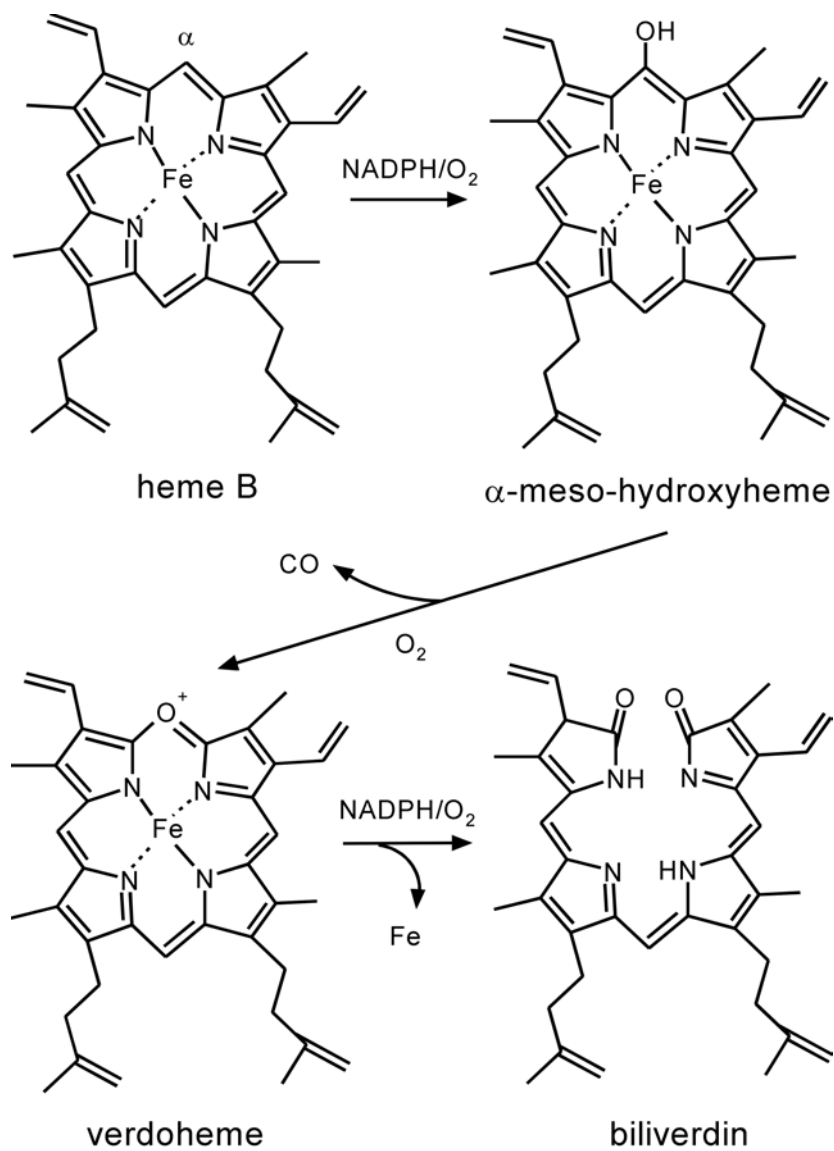
Figure 7. Heme degradation as catalyzed by the heme mono-oxygenases, e.g. HmuO of *C. diphtheriae*.

3 The present investigation

3.1 The *E. faecalis* respiratory chain

3.1.1 NADH dehydrogenase and menaquinone

Respiratory chains are membrane-bound and in bacteria they are generally composed of several dehydrogenases, a quinone pool and one or several terminal oxidases. The dehydrogenases oxidize substrates such as NADH and succinate, and pass the generated electrons on to the terminal oxidases via quinone. The electrons move from a high to a low energy level (i.e. from a low to a high redox potential), thus releasing energy that can be used to e.g. pump protons across the cytoplasmic membrane. Respiration in *E. faecalis* coupled to oxidative phosphorylation and NADH-consumption was reported 25 years ago (Clarke and Knowles, 1980; Pritchard and Wimpenny, 1978). To define the *E. faecalis* respiratory chain, we search for the three basic components of a minimal respiratory chain: a membrane-associated NADH dehydrogenase, quinone, and a terminal oxidase.

There are several potential respiratory dehydrogenases in *E. faecalis* as deduced from the genome sequence. In addition to a lactate dehydrogenase and a glycerol-3-phosphate dehydrogenase, several NADH oxidizing enzymes can be found. The thoroughly studied NADH peroxidase, NPXase, has been show to exert quinone reductase activity in vitro (Marcinkeviciene and Blanchard, 1995). NPXase catalyses the degradation of hydrogen peroxide, forming water and NAD^+. The NADH oxidase, NOXase (catalyzing the oxidation of NADH by molecular oxygen, forming water and NAD^+) is well characterized (Hoskins *et al*., 1962). In the genome sequence of strain V583, two additional NADH oxidase-encoding genes have been tentatively identified, but no experimental data are available regarding their gene products. These NADH dehydrogenases are predicted to be soluble enzymes: However, they may accept electrons from NADH and use them to reduce menaquinone in the membrane, by direct interaction with menaquinone or via unknown interaction partners.

The automated annotation of the V583 genome sequence performed at TIGR has identified two genes, EF1388 and EF1389,

that may encode membrane-bound NADH oxidases. These genes encode proteins that are similar to subunits E and F, respectively, of the *Escherichia coli* respiratory NADH dehydrogenase I (NDH-I)*. However, genes for other subunits of NDH-I cannot be found in *E. faecalis*. This indicates that NDH-1 is not present in this bacterium. EF1388 and EF1389 seem to be in the same operon as a gene encoding a formate dehydrogenase. This indicates that they encode the NAD-reducing subunits of S-FDH (soluble NAD^+-reducing formate dehydrogenase; Friedebold and Bowien, 1993), an anaerobic fermentative enzyme.

To investigate respiratory activity in *E. faecalis*, and its dependency on heme, we studied NADH oxidation activity in isolated membrane and cytoplasmic fractions, from cells grown with or without added heme in the medium. NADH oxidation activity can be measured spectroscopically as the decrease in absorption at 340 nm. Membrane and cytoplasmic fractions of *E. faecalis* V583 were prepared as described in Paper I. NADH oxidation activity assays were performed at 30° C in 20 mM MOPS/NaCl buffer, pH 7.4. The reaction was initiated by the addition of cell material to the cuvette. NADH oxidation activity in membranes isolated from cells grown in the absence of added hemin was much lower than in membranes from cells grown in medium supplemented with hemin (Figure 8). In contrast, the cytoplasmic fraction from cells grown without hemin showed much higher NADH oxidation activity, than that from cells grown in the presence of hemin. The total NADH oxidizing activity of the cells was essentially independent of hemin.

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* *E. coli* contains two membrane-bound NADH dehydrogenases, NDH-I and NDH-II, encoded by the *nuo* operon and the *ndh* gene, respectively. NDH-I-mediated NADH-oxidation is coupled to translocation of protons across the cell membrane, while NDH-II consists of a single polypeptide and is an enzyme that does not pump protons.
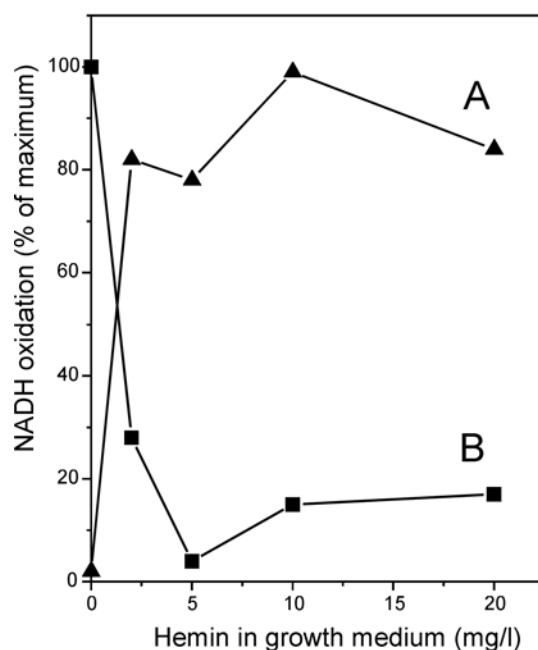
Figure 8. NADH oxidation activity in membrane (A) and cytoplasmic (B) fractions prepared from *E. faecalis* strain V583 grown in the presence of various amounts of added hemin. Activity was calculated as nmoles NADH oxidized per minute and mg protein. 100 % activity was 1.5 nmoles/min*mg for A and 0.11 nmoles/min*mg for B.

We also studied the effect of different inhibitors on NADH oxidation activity of *E. faecalis* membranes. CN⁻ is a structural analogue of dioxygen and, as such, an inhibitor of terminal oxidases. CN⁻ thus affects NADH oxidation if this activity depends on a terminal respiratory oxidase. The NADH oxidation activity in membranes from cells grown in the presence of hemin was found to be sensitive to KCN, while the activity in membranes from cells grown without hemin was less affected (Figure 9). NADH oxidation activities of cytoplasmic fractions showed equal sensitivity to KCN (at 50 mM KCN the activity was 35 % of maximum) whether prepared from cells grown in the presence of added hemin or not. The menasemiquinone analogue 2-heptyl-4-hydroxyquinoline N-oxide (HQNO) inhibited NADH oxidation activity only in

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membranes isolated from cells grown in a medium with added hemin (Figure 10). This suggests that the NADH oxidation activity in membranes isolated from cells grown in the presence of hemin involves a menaquinone reducing activity. The sensitivity of NADH oxidation of these membranes to KCN also indicated that this activity is dependent on a terminal oxidase. We therefore conclude that, if supplied with heme, *E. faecalis* V583 membranes contain an NADH dehydrogenase, which catalyzes the transfer of electrons from NADH to menaquinone. This NADH dehydrogenase constitutes the first segment of a respiratory chain.

Menaquinone is present in *E. faecalis* membranes (Collins and Jones, 1979) and the genome sequence contains genes encoding homologues of all enzymes necessary for menaquinone synthesis, i.e. *menA*, *menBEC* and *menFD*.
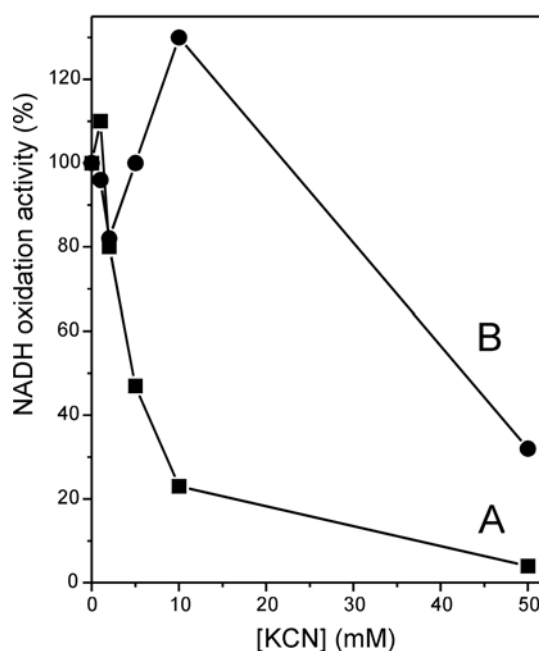
Figure 9. Effect of KCN on NADH oxidation activity in membranes prepared from E. faecalis strain V583 grown in medium with (A) or without (B) added hemin (20 mg/l). The oxidation activity is given as a percentage of the untreated control, and measured as in Figure 8.

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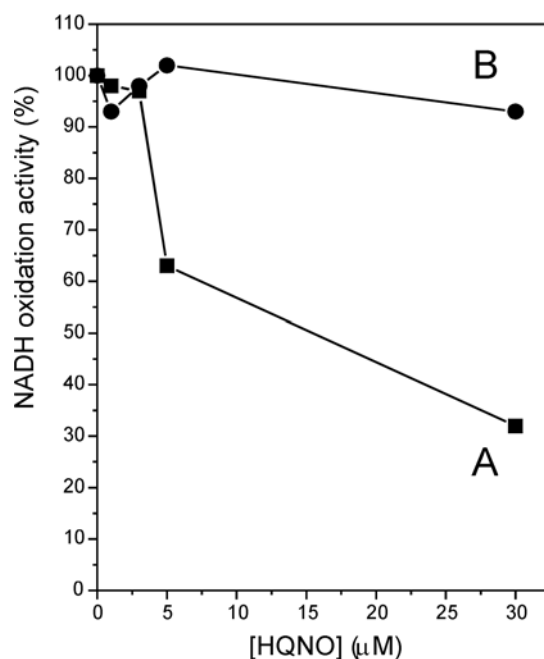
Figure 10. Effect of the menasemiquinone analogue HQNO on NADH oxidation activity in membranes prepared from *E. faecalis* strain V583 grown in medium with (A) or without (B) added hemin (20 mg/l). The oxidation activity is given as percentage of the untreated control, and measured as in Figure 8.

3.1.2   The terminal oxidase

Two candidate terminal oxidases in *E. faecalis* are mentioned in the literature (Huycke, 2002). One is the cytochrome *bd*, which is discussed further on in this section. The other candidate is a fumarate reductase (Huycke *et al.*, 2001). Experimentally, it has been shown that *E. faecalis* cells catalyze fumarate reduction. A membrane-bound quinol-fumarate oxidoreductase (QFR), has been suggested to be present in *E. faecalis* based on the automated annotation of the putative gene EF2556. This gene encodes a protein homologous to

the flavoprotein subunit of the *E. coli* QFR[†]. However, a BLAST search in the NCBI database reveals several more likely homologues, for example the soluble fumarate reductase flavocytochrome c_3 (Fcc3) from *Shewanella frigidimarina* and a soluble fumarate reductase, FRDS1, from *Saccharomyces cerevisiae*. An alignment of the sequences of these proteins and the predicted gene product of EF2556 is shown in Figure 11. The protein encoded by EF2556 seems to belong to an uncharacterized class of fumarate reductases, also found in several other bacteria, e.g. *Lactococcus lactis* and *Listeria monocytogenes*. These reductases show high similarity to the soluble fumarate reductase present in yeast. The major function of this enzyme is thought to be to maintain the $NAD^+/NADH$ balance under anaerobiosis (Enomoto *et al*., 2002). We conclude that EF2556 most likely encodes a fumarate reductase that is not involved in aerobic respiration.

Cytochromes *bd* are found as terminal oxidases in many bacteria, e.g. *E. coli* (Kranz *et al*., 1983), *Azotobacter vinelandii* (Kelly *et al*., 1990) and *B. subtilis* (Winstedt *et al*., 1998). It comprises two protein subunits, A and B, and contains three prosthetic groups: two hemes B and one heme D (Jünemann, 1997). The enzyme catalyzes the two-electron oxidation of quinol and the four-electron reduction of molecular oxygen to yield water. The enzyme does not actively pump protons. Still, cytochrome *bd* participates in the creation of an electrochemical gradient across the membrane. This is obtained by the consumption of protons for water production on the cytoplasmic side of the membrane, while protons are released (following quinol oxidation) on the external side of the membrane.

[†] *E. coli* QFR is composed of four subunits: a flavoprotein (FrdA), an iron-sulfur protein (FrdB) and two hydrophobic polypeptides serving as membrane anchors (FrdC and FrdD).

```
EF2556      1 MKKV-------LMG----------------------------VLSLGLLLGAATG
FRDS1       1 ------------------------------------------------------
Fcc3        1 MKKMNLAVCIATLMGTAGLMGTAVAADNLAEFHVQNQECDSCHTPDGELSNDSLTYENTQ


EF2556     21 CTSDQ----------------------EKAAGKTKASSEK-----------------
FRDS1       1 ------------------------------------------------------
Fcc3       61 CVSCHGTLAEVAETTKHEHYNAHASHFPGEVACTSCHSAHEKSMVYCDSCHSFDFNMPYA


EF2556     39 -----TEATSGASANGYTD-----PSELKDSYDVVIVGSGGAGMTAALQAKEAG-MNPVI
FRDS1       1 -------------------------MSLSPVVVIGTGLAGLAAANELVNKYNIPVTI
Fcc3      121 KKWLRDEPTIAELAKDKSERQAALASAPHDTVDVVVVGSGGAGFSAAISATDSG-AKVIL


EF2556     88 LEKMPVAGGNTIKSSGVNASQTKFQEKEGIKDSNDKFFEDTLKGGKGTNDQELLRYFVD
FRDS1      33 LEKASSIGGNSIKASSGINACGTETQRHFHIPDSPRLFEDDTIKSAKGKGVQELAKLAN
Fcc3      180 LEKEPVIGGNAKLAAGGMNAWTDQQKAKKITDSPELMFEDTVKGGQNINDPALVKVLSS


EF2556    148 HSAEAIDWLD-TKGITISNLTITGGMSEKRTHRPADGSAIGGYLIVDGIVRNVRE------
FRDS1      93 DSPLAIEWLKNEFDLKLDLLAQLGGHSVARTHRSSGKLPPGFELVSALSNNLKKLAETKP
Fcc3      240 HSKDSVDWLYT-AMGADLTDVGMMGGASVNRAHRPTGGAGVGAHVVQVLYDNAVK------


EF2556    201 EKIPLFYDADVTDLVEEN-GQIDGVKVKMKDDKEKTVKAKAVVVTTGGFGANEKLITQYK
FRDS1     153 ELVKINLDSKVVDIHEKD-GSISAVVYEDKNGEKHMVSANDVVFCSGGFGFSKEMLKEYA
Fcc3      293 RNILDLRVNTRGIPVLKDDKGTVKGILVKGMYKGYYWVKADAVILATGGFAKNNERVAKLD


EF2556    260 EEIKNYVITNQEGTTGDGIQMIQKVGGALVDMKEIQIHPT-------VQQSDAFLIGEAV
FRDS1     212 PPIVNLPITNGQQTTGDGQRLLQKLGADYIDMDQIQVHPTGFIDPNDRSSSWKFTAAESL
Fcc3      353 ESLKGFISTNQPGAVGDGLDVAENAGGALKDMQYIQAHPT-------LSVKGGVLVTEAV


EF2556    313 RGEGAILAS-QKGERFVNELDTRDKVSAAINALP---EKSAYLVFDQGVRDRAK-ALDFY
FRDS1     272 RGLGGIILNPITGRRFVNELTTRDVVTAAIQKVCPQEDNRALLVMGEKMYTDLKNNLDFY
Fcc3      406 RGNGAIIVN-REGKRFVNEITTRDKASAAILAQT---GKSAYLIFDDSVRKSLS-KIDKY


EF2556    368 DQKGFVEKGETIEELAEKIG-VPADTLKATIDTWNQDVNAKD--DKQFGRTTGMEADLST
FRDS1     332 MFKKLVQKLTLSQVVSEYNLPTTVAQLCEELQTVSSFTTKADPLGRTVILNEFGSDVTPE
Fcc3      461 IGLGVAPTADSLVKLGKMEG-TDGKALTETVARMNSLVSSGK--DTDFERPN-LPRALNE


EF2556    425 APYYAIKIABGIPHTMGGVKINTKTEVLRE-DGTPIKGLYAAGELTGGLHGQNRIGGNAI
FRDS1     392 TVVEIGEVTEVPFTMGGARINVKAQVTGKNDERLLKGLYAAGEVSGGVHGANRIGGSSL
Fcc3      517 GNYYAIEVTPGVPHTMGGVMIDTKABVVNA-KKQVTPGLYGAGEVTGGVHGANRIGGNAI


EF2556    484 ADIIIYGRQAGTQSAEFASAQK
FRDS1     452 LEGVVFGRTAAESIANDRK---
Fcc3      576 SDILTFGRLAGEEAAKYSKKN-
```

Figure 11. Multiple sequence alignment of the predicted gene product of locus EF2556 in the TIGR database of the V583 genome, and the soluble fumarate reductase FRDS1 of *Saccharomyces cerevisiae* and the tetraheme flavocytochrome fumarate reductase Fcc3 from *Shewanella frigidimarina*. The alignment was generated using the ClustalW WWW service at the European Bioinformatics Institute (EBI) http://www.ebi.ac.uk/clustalw.

Cytochrome *bd* is present in branched respiratory systems, as an alternative quinol oxidase. It generally has a higher affinity for oxygen than the other oxidases, and the enzyme has been suggested to function mainly under conditions of low oxygen tension. The physiological role of cytochrome *bd* seems to vary between organisms. When cytochrome *bd* is absent, *E. coli* has increased temperature sensitivity, show failure to exit stationary phase and production of oxygen radicals is increased (Goldman *et al*., 1996). In *Synechocystis* sp., cytochrome *bd* has been suggested to prevent over-reduction of the plastoquinone pool (Berry *et al*., 2002). It has a similar role, protective rather than energetic, in *A. vinelandii*: to maintain a very low level of oxygen in the cytoplasm in order to protect the nitrogenase (Kelly *et al*., 1990). A cytochrome *bd* has very recently been described in *L. lactis*, where it is essential for long-time survival at low temperatures (Gaudu *et al*., 2002).

When we started our work on enterococci, cytochromes had been reported to be present in *E. faecalis*, but their identity and function remained uncertain. We found that light absorption redox difference spectra of membranes from *E. faecalis* V583 that had been grown in hemin-containing medium show features typical for cytochrome *bd* (Fig. 1 in Paper I). No other cytochromes were detected in the membranes under the experimental conditions used. Membranes from cells grown in the absence of hemin showed no relevant spectral features in the concerned region, indicating an absence of cytochromes under these conditions.
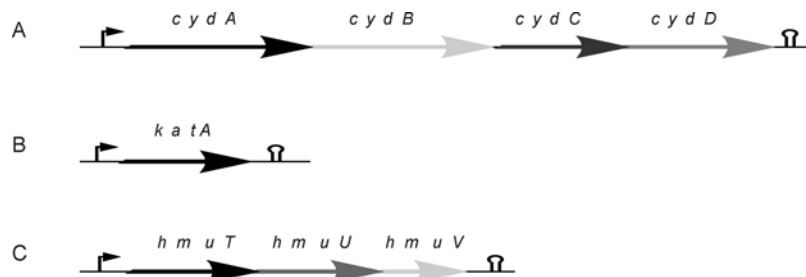
Figure 12. Organization of the *cydABCD* (A), *katA* (B) and putative *hmuTUV* (C) genes in *E. faecalis* strain V583. Hooked arrows and hairpins symbolize putative promoters and transcription termination sites, respectively.

For a genetic analysis of cytochrome *bd*, the amino acid sequence of *B. subtilis* CydA was used to perform a search in the *E. faecalis* genome sequence. The search revealed four genes, similar to *B. subtilis cydABCD*, arranged in an operon (Figure 12). These genes were amplified, using PCR, and cloned in a *B. subtilis-E. coli* shuttle vector. Expressed in *B. subtilis*, *E. faecalis cydABCD* complemented a cytochrome *bd* deficient mutant strain (Paper I).

From the available information, we can now describe the aerobic respiratory chain of *E. faecalis* (Figure 13), consisting of a NADH dehydrogenase, the menaquinone pool and cytochrome *bd*. The relevance of a respiratory chain in *E. faecalis* can be discussed. Maintaining a proper NAD^+/NADH ratio in the cell, i.e. being able to oxidize NADH, is a problem for bacteria relying on a purely fermentative metabolism. Re-oxidation of NADH in these bacteria is mainly performed by the NADH-dependent pyruvate reductase, producing lactate. The presence of an active respiratory chain most likely serves to balance the NAD^+/NADH ratio in a more energy and efficient way as compared to pure fermentation.
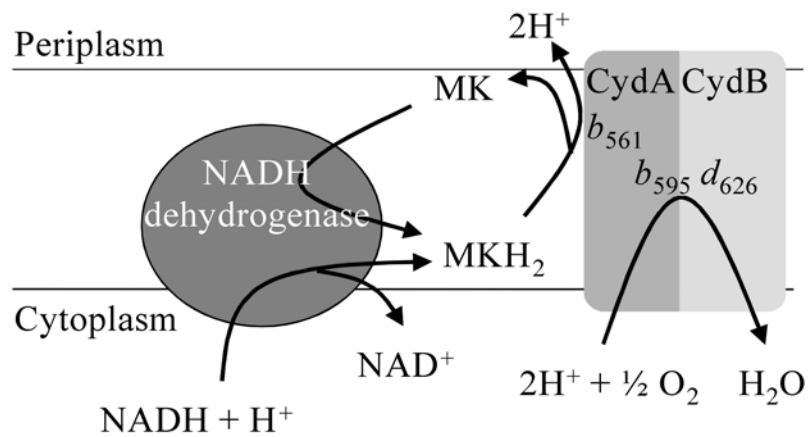
Figure 13. Schematic representation of the suggested respiratory chain of *E. faecalis* strain V583.

3.2 Oxidative stress

3.2.1 The nature and consequences of oxidative stress

Production of free oxygen radicals is an unavoidable consequence of cellular life in an oxic environment. Free radicals are molecules with unpaired electrons, and in most cases they are highly unstable and reactive. They tend to initiate branched chain reactions that may be harmful for the living organism. The most important radical oxygen species are superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($HO^{\cdot}$). Together with hydrogen peroxide (H_2O_2), they are commonly referred to as **reactive oxygen species** (ROS). ROS are normally kept at a low steady-state level by cellular detoxification systems. Oxidative stress occurs when the production of ROS surpasses the capacity of detoxification. This triggers diverse cellular defenses necessary to limit and repair damage on essential cellular components.

Molecular oxygen, O_2, diffuses freely across membranes. In its common, triplet, state oxygen can only accept one-electron transfers.

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Since most reduced molecules in the cell, such as NADH, are obligate two-electron donors, they do not react directly with molecular oxygen. However, several cellular enzymes capable of single-electron transfer exist, especially as parts of respiratory chains. Among the most studied are NADH dehydrogenase II, succinate dehydrogenase and fumarate reductase of *E. coli*. These enzymes contain flavin groups that can be auto-oxidized in the presence of molecular oxygen, creating $O_2^{\cdot-}$ and H_2O_2 (Messner and Imlay, 1999; Messner and Imlay, 2002). The rate of auto-oxidation is normally low, since these flavin-containing enzymes are kept oxidized by their normal substrates. In the presence of inhibitors of electron transfer or a defective respiratory chain, however, the production of ROS can be augmented.

The hydroxyl radical is produced from hydrogen peroxide and catalytic amounts of ferrous iron in the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$). Ferrous iron is regenerated by the action of superoxide anion ($Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$). The two reactions are together known as the Haber-Weiss cycle (the Fe-ion cycles between oxidation state II and III; the net reaction is $H_2O_2 + O_2^{\cdot-} \rightarrow OH^- + HO^{\cdot} + O_2$). The hydroxyl radical is the most reactive of the reactive oxygen species and therefore the most toxic one.

Cell permeability differs somewhat for the different ROS. Superoxide anion has a pKa = 4.8 and diffuses very poorly if at all through membranes at physiological pH, due to its charge (Storz and Imlay, 1999). Biological membranes are permeable to the uncharged hydrogen peroxide molecule. There are, however, several arguments suggesting that membranes only allow slow hydrogen peroxide diffusion (Seaver and Imlay, 2001). Due to the high reactivity of the hydroxyl radical, it is not relevant to discuss its diffusion rate in biological systems.

The superoxide anion exercises a toxic effect by oxidatively destroying iron-sulfur clusters, thus provoking cluster disintegration and loss of enzyme function. Cluster disintegration releases iron, which through the Fenton reaction may aggravate the oxidative stress. Hydrogen peroxide probably exerts little direct toxicity. It can oxidize thiol groups in proteins, hence inactivating enzymes that depend on cysteine residues for activity. However, high toxicity occur from the hydroxyl radical formed when H_2O_2 reacts with iron. The hydroxyl radical rapidly reacts with most biomolecules. DNA is vulnerable to $HO^{\cdot}$ oxidation, and DNA damage is probably the

largest contributing factor to cell death following exposure to the HO˙-generating molecule hydrogen peroxide (Imlay and Linn, 1988).

Cellular defenses against ROS are numerous. Many organisms display redundancy for one or several of these systems, thus illustrating the importance of oxygen detoxification. Superoxide anions are converted to hydrogen peroxide and molecular oxygen by a class of enzymes named superoxide dismutases (SODs). Hydrogen peroxide is subsequently degraded by one of several enzymatic systems: the glutathione/glutathione reductase system, the thioredoxin/thioredoxin reductase system, NADH peroxidase and catalase.

In *E. faecalis*, copious amounts of hydrogen peroxide are produced under certain growth conditions (Huycke *et al*., 1996). An H_2O_2-forming NADH oxidase has been briefly described in *E. faecalis* (Schmidt *et al*., 1986). In addition, the presence of reduced menaquinones in the cell membrane has been incriminated as a cause of hydrogen peroxide production (Huycke *et al*., 2001). This production is attenuated in the presence of a functional respiratory chain. As mentioned in section 3.1, an *E. faecalis* hydrogen peroxide degrading NADH peroxidase has been characterized (Claiborne *et al*., 1992; Marcinkeviciene and Blanchard, 1995; Ross and Claiborne, 1997; Zitzelsberger *et al*., 1983), as well as a glutathione/glutathione reductase system (Patel *et al*., 1998). In addition, sequence similarity data indicates the presence of an alkyl hydroperoxidase, AdhC. However, we expect catalase to be an important hydrogen peroxide-degrading activity when heme is present in the growth medium. The *E. faecalis* catalase is discussed in section 4.3, as well as in Paper II and Paper IV.

3.2.2 Oxidative stress in inter-species warfare

Many researchers active in the field of bacterial pathogenicity are interested in oxidative stress, due to the "respiratory burst" performed by human phagocytes. Upon phagocytosis of a bacterium, the neutrophils unleash a magnificent production of superoxide anions, mediated by an NADPH-oxidase located in the phagosomal membrane (see Hampton *et al*., 1998, for a review on this subject). Due to the low pH of the phagosome, some of the superoxide anions are readily dismutated into hydrogen peroxide, reaching concentrations that have been stated to be as high as 100 mM H_2O_2

(Huycke *et al*., 2002; Roos and Winterbourn, 2002). The oxidative stress thus produced was long thought to be the main cause of bacterial death inside the phagosome. In support of this, it was observed that neutrophils from patients deficient in the NADPH-oxidase[‡] fail to kill many pathogens. The current view is that the main part of the produced hydrogen peroxide is further transformed into hypochlorous acid, HOCl, by the enzyme myeloperoxidase. Hypochlorous acid is an extremely toxic non-radical oxidant, which readily permeates bacterial cells. Since H_2O_2 serves as substrate in the reaction producing HOCL, the actual H_2O_2 concentration in the phagosome is probably much lower than the stated 100 mM.

Lately, a theory has been presented in which the respiratory burst merely serves as a signal for release of protease-loaded granules into the phagosome, and to increase ionic strength of the phagosome in order to solubilize the proteases (Reeves *et al*., 2002). The actual situation probably lies somewhere in between, i.e. the oxidative stress inflicted by the hypochlorous acid and the proteases working together to finish off the trapped pathogen. This may be the explanation for contrasting reports on the importance of catalases and superoxide dismutases for bacterial intra-neutrophil survival. Nevertheless, enzymes protecting against oxidative stress are often important bacterial virulence factors, e.g. in *Staphylococcus aureus*, *Candida albicans* and *Helicobacter pylori* (Clements and Foster, 1999; McGee and Mobley, 1999; Wysong *et al*., 1998).

Microbes also use oxidative stress, especially hydrogen peroxide production, as a weapon in inter-microbial competition (van de Guchte *et al*., 2001). Strains resistant to high levels of hydrogen peroxide may produce large amounts of H_2O_2, which will diffuse into the surrounding medium and efficiently suppress growth of less resistant strains. In the resistant strains, intracellular concentrations of hydrogen peroxide are kept at a tolerable level by enzymatic degradation. The lactic acid bacteria, among which we find *E. faecalis*, are well-known for this behavior, and it is exploited in the lactic acid fermentation of food and beverages, as well as in the production of probiotics.

The high levels of hydrogen peroxide production by *E. faecalis* in vitro, have gained some interest from medical microbiologists. Hydrogen peroxide can act as a α-hemolysin (Barnard and Stinson,

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[‡] This genetic disorder is called chronic granulomatous disease (CGD).

1996) and has also been implicated as a possible cause of colorectal cancer (Huycke *et al*., 2002). *E. faecalis* has been shown, in an animal model, to produce superoxide anions, hydrogen peroxide and hydroxyl radicals when colonized in the intestinal tract. Huycke and coworkers have demonstrated DNA damage in luminal cells from rats colonized with *E. faecalis*. DNA damage was less extensive in luminal cells from rats infected by a strain with attenuated production of oxygen radicals than in cells from rats infected with the wild-type strain.

3.2.3   Catalase

Catalases (EC: 1.11.1.6) are widely distributed among all aerobically living organisms. They catalyze the dismutation of hydrogen peroxide into dioxygen and water. Catalases are classified into three distinct subgroups: the **typical** or **true catalases**, the **catalase-peroxidases** and the **manganese catalases** (Zamocky and Koller, 1999). The first two groups use heme as a cofactor while the manganese catalases have a manganese ion in the active site. Several bacterial manganese catalases have been characterized, e.g. that of *Thermus sp.*(Kagawa *et al*., 1999), *Lactobacillus plantarum* (Igarashi *et al*., 1996) and *Salmonella enterica* (Robbe-Saule *et al*., 2001).

True catalases are usually homo-tetramers, with a subunit size of about 60 kDa. Each subunit contains one heme group. Commonly, this is a heme B, but for example the *E. coli* hydroperoxidase II (HPII) contains heme D (Loewen and Switala, 1986). Crystal structures are available for at least six true catalases. One of these is the *Micrococcus lysodeikticus* catalase (Figure 14). Typically, each subunit forms a globular domain with an extended N-terminal arm. The heme-accommodating cavity is deeply embedded in the protein and lined with highly conserved amino acid residues. The holoprotein is composed of four such globular, heme-containing subunits, each folded around the extended N-terminal arm of its neighbor. This organization makes it likely that the heme group is inserted into the monomer prior to subunit assembly. Little experimental data is available, however, concerning the assembly of catalases (Prakash *et al*., 2002; Putnam *et al*., 2000; Sevinc *et al*., 1998).

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Figure 14. Subunit of the *Micrococcus lysodeikticus* catalase (Murshudov et al., 2002). The heme, shown in space filling, is located in the middle of the globular part of the subunit. The N-terminal threading arm, involved in dimerisation, is seen in the lower part of the image. The protruding loop to the right is probably involved in interactions between the subunits of the tetrameric protein.

True catalases, with a turnover number (K_{cat}) of 40 000 000 per second, are among the most rapid enzymes known. This high catalytic rate can be attained due to the presence of separate inlet and outlet channels in the enzyme. This prevents the leaving product from blocking the access of substrates to the active site. The reaction mechanism is rather well understood. The first event, upon of the substrate, is the production of one molecule of water and the formation of an oxyferryl protein species called Compound I. In Compound I, iron is in the rare Fe(V)-form. A second substrate molecule reduces Compound I, thus producing a second water molecule and molecular oxygen. Incomplete reduction of Compound I may cause the formation of an inactive Fe(IV)-form of the catalase, designated Compound II. Compound II is thought to be slowly reduced back to ground-state catalase by NADPH. NADPH can sometimes be found, depending on the method of purification, as a

cofactor in many catalases (Gouet *et al*., 1995; Murshudov *et al*., 2002; Putnam *et al*., 2000).

E. faecalis catalase activity was briefly described in the literature in the early 1980'ies (Pugh and Knowles, 1982; Pugh and Knowles, 1983). In line with our work on hemoproteins in *E. faecalis*, we performed a characterization of this enzyme (Paper II). Cloning of the gene, *katA* (Figure 12), and homologous and heterologous expression, purification and biochemical characterization allowed identification and description of the KatA enzyme.

The *E. faecalis* catalase is a soluble, cytoplasmic enzyme. Light absorption spectroscopy and HPLC analysis (paper II) identified heme B as the prosthetic group. The monomer mass of KatA is approximately 54 kDa as determined by mass spectrometry (our unpublished data). Due to the similarities in size and sequence, it can be expected that the *E. faecalis* catalase has an overall structure similar to that of *E. coli* HPII and *M. lysodeikticus* catalase.

Using immunological methods, no KatA polypeptide could be detected in *E. faecalis* grown under oxic culture conditions in the absence of heme (Paper II). This suggests that synthesis of KatA apoprotein is repressed, or that the KatA protein is very rapidly degraded, in the absence of heme. Pugh *et al.* (Pugh and Knowles, 1983) have suggested that KatA apoprotein synthesis is induced by aeration alone, independent of the presence of hemin in the growth medium. This suggestion was based on the rapid (within minutes) appearance of active catalase in cells grown without hemin, after the addition of hemin to the growth medium. Given the localization of heme in catalase and the overall organization of the holoprotein, it seems unlikely that the apoprotein would have a stable fold without heme. This would imply that the *katA* gene is expressed under aerobic conditions even in the absence of hemin, but that the apoprotein is not correctly folded and therefore degraded. This conclusion is supported by the finding that only very low levels of KatA antigen can be detected in the absence of heme, even when KatA is expressed from a constitutive promoter, (Paper II).

3.3 Heme uptake by *E. faecalis*

3.3.1 Heme uptake by *E. faecalis*

E. faecalis can take up and use heme from several different sources, e.g. hemin, haematin, blood (Ritchey and Seeley, 1974; Sijpesteijn, 1970) and hemoglobin (own unpublished data). Rather high concentrations of hemin in the growth medium (>5 μM) are necessary to achieve maximal production of catalase (Paper II). This may indicate a low affinity of the uptake system for free heme. Hemin was supplied from a stock solution prepared with water, detergent and NaOH, or dissolved in dimethyl sulfoxide (DMSO). The type of solvent did not affect uptake (own observations, data not shown). The low solubility of hemin in water at physiological pH can be one reason for the poor uptake. Additionally, hemin may be taken up by a transporter specialized for another substrate, e.g. hemoglobin, having only low affinity for the isolated heme molecule. Currently, it is not known by which mechanisms *E. faecalis* cells take up heme from the surrounding medium.

3.3.2 The Hmu system

The Hmu (Hemin utilization) system belongs to the ABC (ATP-Binding Cassette) family of transporters. Although first identified in the human pathogen *Yersinia enterocolitica* (Stojiljkovic and Hantke, 1994), the Hmu transport system has largely been studied in the plague bacterium *Y. pestis* (Thompson *et al.*, 1999). It allows the use of free hemin, as well as various hemoproteins, as a source of iron. The Hmu system has later been found also in other Gram-negative bacteria, e.g. *Shigella dysenteriae* (Mills and Payne, 1995) and *E. coli* (Torres and Payne, 1997).

The *hmu* locus of *Y. enterocolitica* comprises eight open reading frames, of which *hmuR*, *hmuT*, *hmuU* and *hmuV* encode proteins with known functions. The system is dependent on TonB, a ubiquitous transport protein present in Gram-negative bacteria. According to a model proposed by Wandersman and Stojiljkovic, 2000 (see Figure 15), heme binds to the outer membrane receptor HmuR. The binding is followed by a TonB-mediated change of receptor affinity, leading to the transfer and release of heme to the periplasm. A soluble

protein, HmuT, binds the heme-group in the periplasm and delivers it to the inner-membrane permease, HmuU. The energy necessary for the transport of heme across the cytoplasmic membrane is provided by HmuV-mediated ATP hydrolysis. In addition to the genes encoding these functionally well defined proteins, the locus contains the *hmuS* gene, which encodes a protein with unknown function. HmuS has been proposed to have heme-detoxifying properties or a role in the utilization of certain hemoproteins (Stojiljkovic and Hantke, 1994; Thompson *et al*., 1999; Wilks, 2001; Wyckoff *et al*., 1998).
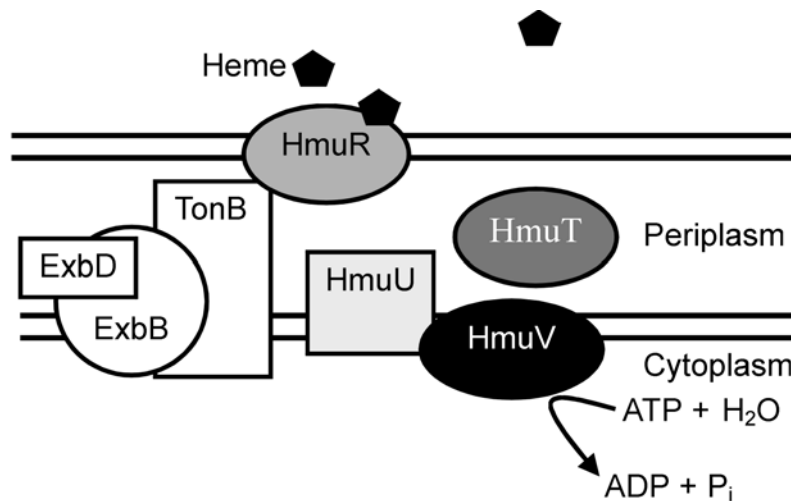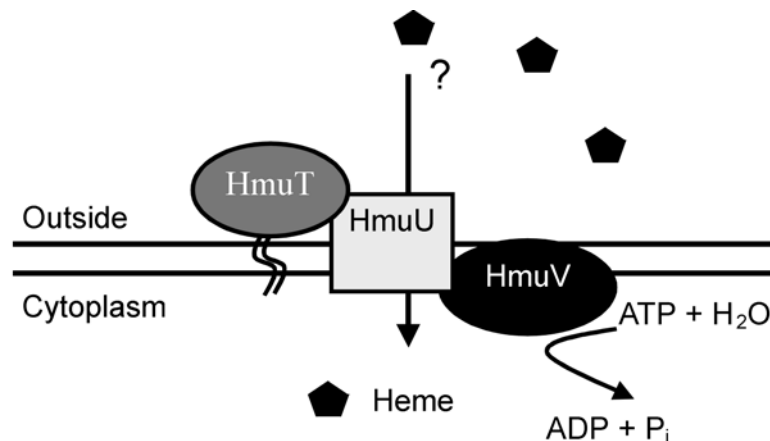

Figure 15. The HmuRTUV transport system of *Yersinia enterocolitica*, figure adapted from Wandersman *et al.*, 2000.

Recently, an *hmu* locus was identified for the first time in a gram-positive bacterium, *Corynebacterium diphtheriae* (Drazek *et al*., 2000). It contains the *hmuTUV* genes, while the genes *hmuR* and *hmuS* are absent. HmuT is attached to the cytoplasmic membrane of *C. diphtheriae* by a lipid anchor and most likely functions as a cell surface heme-binding receptor (Figure 16). *C. diphtheriae* HmuU shows extensive sequence similarity to other described bacterial hemin permeases and probably mediates hemin transport across the

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bacterial membrane. The typical ABC organization of the transport protein is made complete by HmuV, the putative ATPase.

Figure 16. The HmuTUV transport system of *Corynebacterium diphtheriae*, figure adapted from Drazek *et al.*, 2000.

3.3.3   Hmu proteins in *E. faecalis*

Identification of genes specifically corresponding to the *C. diphtheriae hmuTUV* genes in the *E. faecalis* genome is difficult because of the high sequence similarity among all ABC-type transporters. However, the EF1639, EF1640 and EF1641 genes of the V583 genome sequence constitute a putative *hmuTUV* operon (Figure 12). The operon is arranged in the same manner as in *C. diphtheriae*. The neighboring genes do not seem to be involved in heme synthesis, degradation or transport. An *hmuO* homologue is not found in the available *E. faecalis* genome sequence.

3.4   <u>Non-heme porphyrins</u>

### 3.4.1   Introduction and chemistry of porphyrins

One of the particularities of *E. faecalis* hemoprotein synthesis is the incorporation of exogenously supplied heme into native, and possibly also heterologously expressed, proteins. Early in our work it became obvious that this provides an opportunity for the in vivo synthesis of non-natural proteins by feeding *E. faecalis* cells modified heme, e.g. heme analogues.

We concentrated on the synthesis of hemoproteins containing heme-analogues, i.e. porphyrins other than heme. When present, the metal is the reactive center of the porphyrins. It also determines the coordination chemistry of the molecule, i.e. the number and nature of possible protein ligands. Heme carries a chelated Fe-ion, while another famous porphyrin, chlorophyll, is a Mg^{2+}-containing porphyrin. The type of chelated metal, together with variations in the porphyrin periphery, are the basis for the functional differences between heme and chlorophyll: heme is specialized in oxygen transport/reduction and electron transfer, while chlorophyll is involved in light energy harvest.

It is not always possible to explain the molecular basis for differences in functional properties of porphyrins. However, the influence of some very basic aspects of porphyrin structure on functionality are known. The aromatic ring structure is responsible for the great stability of the porphyrin. As the energetically favored constellation, aromaticity will be preserved through most reactions that porphyrins undergo. A large energy input is necessary to chemically or enzymatically oxidize the ring. The conjugated bond system is responsible for charge equalization during electron transfer reactions and mediates the capture of photons.

The nature of the substituents of the macrocycle governs the shape and size of the molecule. Shape and size are critical factors for the successful incorporation into proteins. The influence of the sidechains on porphyrin reactivity is less apparent. Still, substituents fine-tune properties such as the redox potential and may sterically hinder access to the metal ion for incoming substrates (Milgrom, 1997).

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Overall, it is the nature of the chelated metal ion that is most important in this matter. Heme owes its unique properties to the presence of the Fe-ion capable of reversible binding to ligands, such as oxygen, and cycling between redox states Fe(II), Fe(III), Fe(IV) and Fe(V). In chlorophyll, non-redox active magnesium is active in light collection. Thus, if the metal of a certain porphyrin is replaced with another one, the properties are changed. For example, replacement of the iron ion of heme with a larger metal ion induces structural strain on the porphyrin molecule, causing it to diverge from its planar shape into a more domed constellation. The coordination geometry may also be affected, however it is very difficult to predict in what way. The porphyrin-chelated ferrous ion displays a 6-coordinate octahedral geometry (Figure 17), but when incorporated into a protein, the coordination geometries undergo subtle changes and the heme iron may present a slightly distorted coordination geometry. The distortion of the ligand coordination may have large importance for the enzymatic activity of the protein. Replacement of the ferrous ion with an ion presenting another coordination geometry therefore usually affects reactivity.
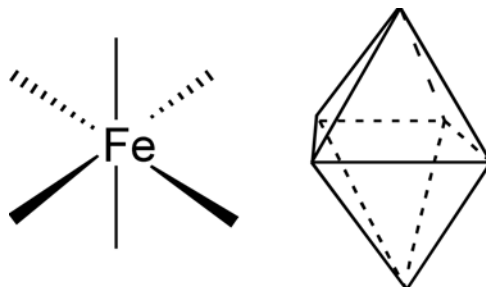
Figure 17. Octahedral coordination geometry of iron.

3.4.2   Antimicrobial activity of porphyrins

The emergence of microbial strains resistant to most commonly applicable antibiotics has drawn attention to the anti-microbial properties of some porphyrins. Two classes of bactericidal

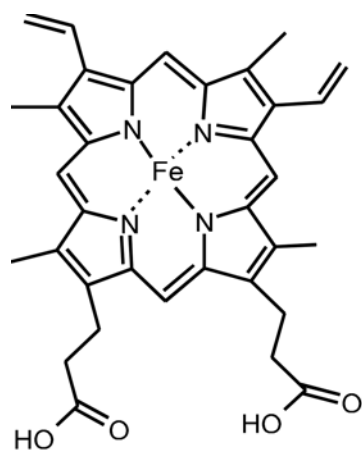porphyrins can be distinguished: the photosensitizers and the heme-analogues.

The photosensitizers are successfully used in photodynamic therapy, PDT, a light-dependent treatment used in the clinic against certain kinds of tumors (Dalla Via and Marciani Magno, 2001). The porphyrins used in PDT are usually given to patients by intravenous injection. The compounds accumulate in certain tumor tissues where they are subsequently activated when light is shone onto the tumor. The light is applied by highly selective methods, thus reducing toxicity for the healthy tissue surrounding the tumor.

Among the most studied photosensitizers are deuteroporphyrin and protoporphyrin IX. In the presence of light, these non-metal porphyrins show high bactericidal activity against many gram-positive and some gram-negative bacteria (Nitzan et al., 1992). The exerted killing depends on the presence of an outer membrane and how well the photosensitizer is able to adhere to the bacterium. Light activation of the porphyrin is thought to cause damage to the cytoplasmic membrane, which results in loss of cell electrolytes and oxidative damage. Hemin administrated in large doses seems to cause modest cell killing by a similar mechanism but independent of light activation. However, an extensive bactericidal effect can be obtained by combined use of hemin and deuteroporphyrin (Malik et al., 1990). The two porphyrins are believed to form a potent complex, capable of inducing strong light-independent oxidative reactions.
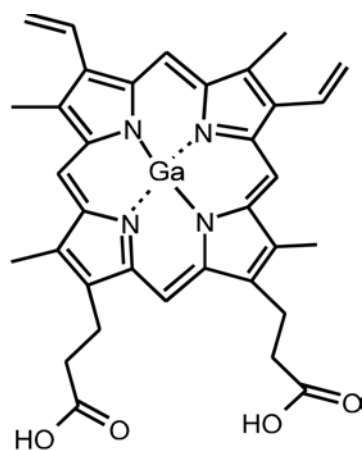
The heme analogues probably act by a different mechanism. It has been suggested that their structural similarity to heme ("mistaken identity") allows these porphyrins to be transported into the cell and inserted into heme proteins (Stojiljkovic et al., 1999). However, the heme analogues could also act by a mechanism similar to that of the photosensitizers. If the porphyrins are mistaken for heme in the bacterial cell, numerous cell proteins loose their biological activity, when the normal heme-prosthetic has been replaced by functionally inert porphyrins. The most severe cell damage probably results from the inactivation of components in the respiratory chain. The resulting incomplete oxidation of electron donors in the membrane in turn causes the formation of ROS. In accordance with this, bacterial mutants deficient in SODs and catalases are hyper-sensitive to heme analogues (Stojiljkovic et al., 1999). Knowing the likely mechanism of action of the heme analogues, one can predict that only respiring bacteria, capable of heme uptake, are sensitive to heme analogues.

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Indeed, strict anaerobes, anaerobically grown bacteria and fermenting bacteria as well as strains lacking the ability to take up heme, are resistant to heme analogues (Stojiljkovic *et al.*, 1999). The "mistaken identity" theory of action is furthermore supported by the observation that administration of hemin at high concentration abolishes the toxic effects of heme analogues.
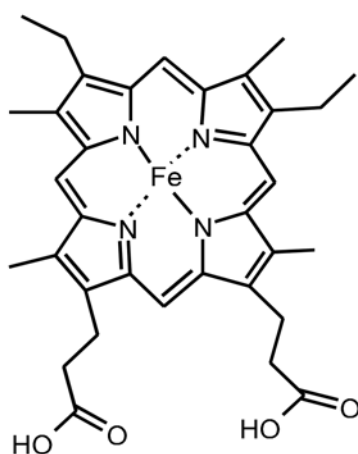
Compounds known to exert this kind of toxicity are e.g. many kinds of non-iron metallo-protoporphyrin IX, some metal containing mesoporphyrins (Figure 18) and to a lesser degree gallium chlorine e_6. The corresponding metal-free porphyrins, and the metals administrated alone, are less toxic. Very low toxicity has been observed with compounds differing largely in structure from protoporphyrin IX, e.g. gallium porphine.

Figure 18. Fisher drawing of heme B (for reference), GaPPIX (gallium protoporphyrin IX, a metallo-PPIX), FeMesoPP (iron mesoporphyrin) and gallium porphine.

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The practical implications of anti-microbial activity of porphyrins are promising. For example, excellent results have been obtained using a mixture of deuteroporphyrin and hemin for treatment of burn-wound *Staphylococcus aureus* infections in an animal model (Orenstein *et al*., 1997). The efficiency of heme-analogues has not, to our knowledge, yet been tested in animal models. Anti-microbial activity , however, has been shown against a large number of clinically important pathogens, while toxicity for at least some mammalian cells in vitro is low (Stojiljkovic *et al*., 1999).

3.4.3   Metalloporphyrins used in this study

We have made used of different non-heme porphyrins to synthesize artificial catalases (Table I). Most of them are metallo-protoporphyrin IX's, which contain a metal other than iron. Our studies started with the use of **gallium protoporphyrin IX**, a molecule being highly toxic for many bacteria but not for *Enterococcus sp.*. The Ga^{3+} ion has an ionic radius that is almost identical to that of Fe^{2+} (76 pm compared to 75 pm) [§]. Thus GaPPIX [**] and heme are similar in structure. The metal ion of GaPPIX is radically different from the one of heme, though, as it does not take on any other oxidation states. This property is shared also by **zinc protoporphyrin IX**, **magnesium protoporphyrin IX** and **tin protoporphyrin IX**. Consequently, no redox activity can be expected to be associated with these compounds. The Zn^{2+}, Mg^{2+} and $Sn4^{+}$ ions are slightly larger than Fe^{2+}: 88, 86 and 83 pm respectively. This bulkiness induces steric strain on the tetrapyrrole, causing the macrocycle to bend slightly to accommodate the metal ion.

In addition to these non-redox compounds, experiments were also conducted with a set of porphyrins containing redox-active metal: **nickel protoporphyrin IX**, **copper protoporphyrin IX, cobalt protoporphyrin IX**, and **manganese protoporphyrin IX**. Out of these porphyrins, only CoPPIX contains a metal (Co^{3+}) of approximately the same ionic radius as Fe^{2+} (68 pm). The metal ions of NiPPIX and CuPPIX have ionic radii that are larger than that of

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[§] All ionic radii are given as Shannon ionic radii, in picometers, for the 6-coordinate, low-spin (when applicable) ion.
[**] The metallo-protoporphyrin IXs will be abbreviated by the chemical symbol of the metal followed by protoporphyrin IX in short (PPIX), e.g. GaPPIX for gallium protoporphyrin IX.

Fe^{2+} (83 and 87 pm, respectively), whereas the metal ion of MnPPIX is slightly smaller (72 pm). Note that also a small metal ion may induce some bending of the tetrapyrrole macrocycle, due to the metal-nitrogen bonds.

We were also curious about the effects of variations in substituents of the porphyrin macrocycle and therefore included **iron mesoporphyrin IX** in our study. The mesoporphyrins differ from the protoporphyrins by the presence of two ethyl groups instead of the vinyl groups at two of the β-carbons. **Palladium mesoporphyrin IX** and **ruthenium mesoporphyrin IX** were also tested. The redox-active palladium ion has a very large radius, 100 pm, and the porphyrin of palladium is probably considerably distorted. Ru-mesoporphyrin is a particularly interesting heme-analogue. The ionic radius of Ru^{2+}, 82 pm, allows a planar constellation of the porphyrin, and an ruthenium ion can donate a single electron upon excitation with a laser flash.

Table 1. Some biological and chemical properties of porphyrins used in this study.

Porphyrin	Ion charge	Redox activity[††]	Ionic radius[‡‡]	Metal coordination number	Toxicity[§§]	KatA prod.[***]
CoPPIX	3+	Y	69	4, 6, 8	N	Y
CuPPIX	2+	Y	87	4 (6)	N	Y
FeMesoPP	2+	Y	75	4, 6, 8	N	Y
FePPIX (heme)	2+	Y	75	4, 6, 8	N	Y
GaPPIX	3+	N	76	4, 6	N	Y
MgPPIX	2+	N	86	4, 6, 8	N	Y
MnPPIX	3+	Y	72	4, 6, 8	Y	N
NiPPIX	2+	Y	83	4, 6	N	?
PdMesoPP	2+	Y	100	4, 6	Y	N
PPIX	-	-	-	-	N	Y
RuMesoPP	4+	N	82	6	N	Y
SnPPIX	4+	N	83	4, 6, 8	N	Y
ZnPPIX	2+	N	88	4, 6, 8	N	Y

[††] Redox activity can be expected from the metal ion, yes (Y) or no (N).
[‡‡] All ionic radii are given as Shannon ionic radii, in pm for the 6-coordinate, low-spin (when applicable) ion.
[§§] Toxicity against *E. faecalis* have been observed, yes (Y) or no (N).
[***] When the porphyrin is administrated in the growth medium, catalase production has been observed, yes (Y) or no (N).

3.4.4   In vivo synthesis of non-natural heme-proteins

As *E. faecalis* does not depend on hemoproteins for growth under aerobic conditions, it could be expected that this bacterium would be resistant to the toxic effect of heme analogues. Experimental results promptly showed that this was indeed the case. *E. faecalis* was found resistant against many metalloporphyrins being bacteriocidal for other gram-positive bacteria (Table 1 and Paper III). The assays were conducted under conditions where *E. faecalis* is known to take up heme from the growth medium. Consequently, it was predicted that the enterococcal cells were taking up the heme analogues and incorporating them into endogenous hemoproteins. Since *E. faecalis* mainly relies on a fermentative energy metabolism, little or no cellular damage occurs from the non-functional hemoproteins.

We have exploited this unique tolerance of *E. faecalis* cells to establish a procedure that allows incorporation of heme-analogues into KatA. A strain over-producing the *E. faecalis* His_6-tagged catalase was grown in medium containing the desired heme-analogue. Purification of KatA from the cultured cells and analysis of the proteins showed that they contained a non-heme porphyrin.

Using this method, we have synthesized a collection of artificial catalases containing a heme-analogue (in our case GaPPIX, CoPPIX, SnPPIX, ZnPPIX, RuMesoPP and FeMesoPP) in the place of heme (Paper III). Only one of these catalases, the FeMesoPP-containing catalase, showed significant catalase activity.

Catalase isolated from cells grown in the presence of protoporphyrin IX contained metal-free protoporphyrin IX. This was surprising, since we expected protoporphyrin IX to be converted to heme by HemH. The PPIX-containing catalase showed some activity, about 16 % compared to the native KatA. Two of the other artificial catalases, produced with CuPPIX and MgPPIX in the growth medium, were also identified as PPIX-containing catalases. This indicates that the metal ion of these porphyrins was lost or removed during culture, uptake or transport inside the cells. The PPIX-containing catalases produced from CuPPIX and MgPPIX were devoid of activity, a phenomenon we cannot explain at this time.

The synthesis of PPIX-containing catalase in a bacterium that contains a *hemH* gene was unexpected. Possibly the gene is inactivated, not expressed or encodes a protein with sequence similarity to ferrochelatase but with a different function.

The described method for production of artificial can possibly be used for synthesis of a great variety of complex soluble and membrane-bound artificial hemoproteins. Artificial hemoproteins are potentially useful in biotechnology and basic research. Hitherto, such molecules have not been possible to synthesize.

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4 Future directions

In this PhD thesis work, the *E. faecalis* cell has been established as a convenient model system for studies on heme and heme-proteins. This easily cultured gram-positive bacterium probably allows systematic knockout of genes encoding proteins essential for heme-dependent organisms. Knockouts of hemoproteins present in *E. faecalis* will tell us more about the physiology of this bacterium. Furthermore, the presence of native hemoproteins both in the cytoplasm (catalase) and in the cytoplasmic membrane (cytochrome *bd*) allows the study of the compartmentalization of heme.

The presence of a respiratory chain and a heme-dependent catalase in *E. faecalis* changes the current view of energy metabolism and pathogenicity of this bacterium. Further studies of the aerobic heme-dependent biology of the enterococci may reveal important information regarding *E. faecalis* virulence and assist in fighting the emergence of multi-resistant pathogenic strains.

The unique capacity of *E. faecalis* to synthesize non-essential hemoproteins has been successfully applied for the synthesis of artificial catalases. The study of a range of artificial catalases with various spectroscopic and enzymatic properties will add information about the complex enzymatic mechanism of catalases. This illustrates the useful properties of *E. faecalis* as an expression system for hemoproteins. Most likely, many different artificial hemoproteins, both soluble and membrane-bound, can be produced in *E. faecalis*, adding knowledge about different processes such as drug degradation and electron transport.

The *E. faecalis hemH*-gene is intriguing. Why does not the *E. faecalis* cell transform protoporphyrin IX into heme? Maybe the *hemH* gene is not expressed, or possibly it encodes a protein with sequence similarity to ferrochelatase but with a different function.

5 Summary of papers

5.1 Paper I.

Analysis of membranes by light absorption difference spectroscopy showed that *E. faecalis* cells grown in the presence of heme contained a cytochrome *bd*. Putative cytochrome *bd*-encoding genes, *cydABCD*, were identified in the *E. faecalis* genome sequence. These genes, cloned and expressed in *B. subtilis*, complemented a cytochrome *bd*-deficient *B. subtilis* strain. The results indicate that *E. faecalis* can produce a functional cytochrome *bd*-type quinol oxidase and has the capacity of aerobic respiration if supplied with exogenous heme.

5.2 Paper II

E. faecalis is in the older literature referred to as devoid of catalase. However, catalase production in this bacterium can be observed when heme is supplied in the growth medium. We identified the catalase-encoding gene, *katA*, and cloned it. A catalase-deficient *B. subtilis* mutant was complemented by the *E. faecalis katA* gene. A histidyl-tagged version of the KatA protein was purified and characterized. The enzyme contains one molecule of protoheme IX per protein subunit. Production of KatA and catalase activity in *E. faecalis*, is heme-dependent. Our results show that, if supplied with heme, *E. faecalis* can synthesize a true catalase.

5.3 Paper III

We have exploited *E. faecalis* for the in vivo production of catalase containing porphyrins other than heme. If heme-mimicking molecules are added in the growth medium, *E. faecalis* can take them up and integrate them to form stably folded catalase. Several heme analogues, e.g. zinc-protoporphyrin IX, gallium-protoporphyrin IX and iron mesoporphyrin, could thus be incorporated into KatA protein. All resulting proteins were devoid of catalase activity except the iron mesoporphyrin–containing KatA. Addition of metal-free protoporphyrin IX resulted in KatA containing protoporphyrin IX. These results show that *E. faecalis* constitutes a convenient platform for in vivo synthesis of artificial hemoproteins.

5.4   Paper IV

To analyze the role of catalase in *E. faecalis*, the *katA* gene was disrupted by the insertion of a plasmid. The resulting mutant lacks catalase activity and KatA protein. It presented a growth deficiency when cultivated in the presence of heme. We could not, however, find any decrease in resistance to hydrogen peroxide, compared to the parental strain. Until further experiments have been performed, the physiological role of KatA in *E. faecalis* remains uncertain.

6 Sammanfattning på svenska

Hem är en liten molekyl som förekommer som aktiv grupp i många viktiga proteiner, t.ex. hemoglobin, katalas och cytokromer. De hem-innehållande proteinerna är livsnödvändiga för alla djur och växter, samt många bakterier. Kemiskt sett, är hem en **porfyrin** (en annan känd porfyrin är klorofyll) som innehåller en järn-jon.

Bakterien *Enterococcus faecalis* är en vanlig invånare i friska människors och djurs tjocktarm, dock kan den också orsaka svåra sjukdomar. Dessutom är *E. faecalis* ofta resistent mot vanligt använda antibiotika vilket gör dessa sjukdomar svårbehandlade. *E. faecalis* är en av ett litet antal kända organismer som inte behöver hem-proteiner för att överleva. Denna bakterie kan inte själv göra hem, men kan ta upp hem från tillväxtmediet för att sedan sätta in det i det färdiga proteinet.

Vi har beskrivit ett **cytokrom *bd*** som förekommer i *E. faecalis* endast när bakterien har tillgång till hem. Cytokrom *bd* fungerar som terminalt oxidas i bakteriens andningskedja. *E. faecalis* behöver dock inte sin andningskedja för att överleva, eftersom den också kan använda sig av jäsning för sin energimetabolism. Ytterligare ett protein, **katalas**, förekommer i *E. faecalis* endast när det finns hem i tillväxtmediet. Katalas är ett viktig enzym som skyddar cellerna mot fria radikaler. Fria radikaler kan orsaka skador på arvsmassan och därmed ge upphov till cancer. Reaktionsmekanismen för katalas är ganska välkänd, men det finns fortfarande en del oklarheter. Katalas katalyserar nedbrytning av väteperoxid till syrgas och vatten. Vi har renat enzymet från *E. faecalis*, bestämt dess biologiska och kemiska egenskaper samt tagit fram antikroppar mot proteinet. Vi har också utnyttjat *E. faecalis* för att tillverka icke-naturligt katalas. Genom att tillföra **hem-analoger** i tillväxtmediet (molekyler som liknar hem men är lite annorlunda) har vi lyckats tillverka flera olika katalaser, med olika kemiska och biofysikaliska egenskaper. Dessa kan senare t.ex. användas som verktyg för att studera katalasets komplicerade enzymatiska mekanism. Troligen kan också andra hem-innehållande proteiner framställas och modifieras med denna metod, och vi kan på så sätt vinna mer information om viktiga enzymatiska processer, som t.ex. respiration och nedbrytning av läkemedel i vår kropp.

7 Acknowledgements

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