BACTERIAL THIOREDOXIN SYSTEMS AS POTENTIAL DRUG TARGETS IN NOVEL ANTIMICROBIAL THERAPIES

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ABSTRACT

Antimicrobial resistance is a big problem in modern medicine. One of the key components in the combat is the characterization of novel drug targets and the development of new classes of compounds targeting them. In the case of the gastric pathogen *Helicobacter pylori*, increasing resistance necessitates more elaborate and costly treatment-regimes, which cause more side effects than the standard therapy. As for *Bacillus anthracis*, the causative agent of anthrax, naturally occurring or engineered resistance threatens to compromise the treatment of infections caused by this severe pathogen.

The aim of this thesis was to characterize bacterial thioredoxin systems as potential drug targets in bacteria devoid of glutathione. Furthermore, to develop inhibitors that targets these systems. Special emphasis was given to *H. pylori* as a representative for slow growing, chronic, pathogens and *B. anthracis* for rapidly growing pathogens giving rise to fulminant disease.

Paper nr I: We provided crystal structures of *H. pylori* thioredoxin reductase in both the oxidized and reduced forms at 1.7 and 1.45Å resolution, respectively. This information could potentially be used for rational design and optimization of inhibitors.

Paper nr II: We characterized the thioredoxin system of *B. anthracis* and concluded that, despite an apparent redundancy, *B. anthracis* has only one thioredoxin reductase, two thioredoxins and one NrdH-redoxin. Furthermore, thioredoxin 1 was the predominant disulfide reductase and the physiologically relevant electron donor for ribonucleotide reductase. This suggests that the thioredoxin system of *B. anthracis* could be an attractive drug target.

Paper III: We described inhibition of bacterial thioredoxin reductases as a novel antibiotic principle using ebselen. Using pure proteins, we could show that ebselen is a competitive inhibitor of *E. coli* thioredoxin reductase, acting by binding to the C-terminal active site cysteine. We used various *E. coli* strains with gene-deletions in the redox systems to show that the absence of glutathione confers sensitivity to ebselen. We also showed that *H. pylori* and *M. tuberculosis*, both of which naturally lack glutathione, are sensitive to the drug.

Manuscript IV: We studied ebselen and derivatives thereof as inhibitors of *B. anthracis* thioredoxin reductase and tested their antibacterial activity against *B. subtilis*. We could show that selected compounds are potent inhibitors of *B. anthracis* thioredoxin reductase with IC50 values down to 70 nM. Minimal Inhibitory Concentrations (MICs) were down to 0.4 µM (0.12µg/ml) for *B. subtilis* and compounds had a bacteriocidal mode of action. Based on this, we constructed a structure-activity-relationship and concluded that ebselen and derivatives thereof could potentially be used for the treatment of anthrax.

In conclusion, the present thesis deals with the characterization of bacterial thioredoxin systems as potential drug targets - on a biochemical, bacteriological and structural level. The results obtained suggest that bacterial thioredoxin systems are attractive drug targets in bacteria devoid of glutathione and that ebselen might be a viable starting point for drug development. Inhibitors could potentially be used to treat a wide variety of infections including anthrax, gastric ulcers and tuberculosis.


IV. **Gustafsson TN**, Kandasamy K, Engman L, Holmgren A. Ebselen and analogs as inhibitors of Bacillus anthracis thioredoxin reductase – antibacterial properties with a bacteriocidal mode of action. *Manuscript*
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LIST OF ABBREVIATIONS

BSH  Reduced bacillithiol
Cys (C)  Cysteine
C_{max}  Maximal concentration of drug achieved during therapy
DTNB  5,5′-dithiobis-(2-nitrobenzoic acid)
DTT  Dithiothreitol
EbSe  Ebselen (2-phenyl-1,2-benzoiselenazol-3(2H)-one)
ERF-cloning  Exonuclease I dependent Restriction Free cloning
FAD  Flavin adenine dinucleotide
Fe-NrdF  Iron-containing NrdF
GR  Glutathione reductase
Grx  Glutaredoxin
GSH  Reduced glutathione
GSSG  Glutathione disulfide
IMAC  Immobilized metal affinity chromatography
k_{cat}  Turnover number
K_{m}  Substrate concentration for which velocity = V_{max}/2
MBC  Minimum bacteriocidal concentration
MIC  Minimum inhibitory concentration
Mn-NrdF  Manganese-containing NrdF
MSR  Methionine sulfoxide reductase
NADPH  Nicotinamide adenine dinucleotide phosphate (reduced form)
NADP^{+}  Nicotinamide adenine dinucleotide phosphate (oxidized form)
NrdA  Large component of class Ia ribonucleotide reductase
NrdB  Small component of class Ia ribonucleotide reductase
NrdE  Large component of class Ib ribonucleotide reductase
NrdF  Small component of class Ib ribonucleotide reductase
NrdH  Glutaredoxin-like reductant of class Ib ribonucleotide reductase
NrdI  Flavodoxin-like activator of Mn-NrdF
RNR  Ribonucleotide reductase
Ser (S)  Serine
TEV protease  Tobacco Etch Virus protease
Trx  Thioredoxin
TR  Thioredoxin reductase
V_{max}  Maximal velocity
Wt  Wild type
1 INTRODUCTION

1.1 THE PROBLEM OF ANTIMICROBIAL RESISTANCE

One of the big problems in modern medicine is the increasing antimicrobial resistance. Antimicrobial resistance results in increased morbidity, mortality and cost. Additionally, it threatens to compromise our ability to cope with infectious complications normally associated with surgical procedures, cancer treatments, dialysis and transplantations (1).

In an American study, infections with methicillin resistant Staphylococcus aureus (MRSA) were associated with almost three times higher mortality compared to infections with methicillin sensitive S. aureus (MSSA) (2). Furthermore, a recent report suggests that a selected list of resistant bacterial pathogens are responsible for a minimum of 25 000 deaths and increased costs of € 1.5 billion annually in the EU (3).

In “the golden era of antibiotics” resistance was not considered a big problem given that new classes of antibiotics were continuously being introduced, keeping us ahead of resistance. The situation has since changed – few new drugs are being approved, and most of them are small variations of a well known scaffold, targeting a limited set of functions in the bacteria (3–5). This is illustrated by the β-lactams (including the penicillins), a class of antibiotics which has been extensively used and varied over the years (6).

The reasons for the lack of new antibiotics are many but can broadly be divided into financial, scientific and regulatory (7). One important aspect is the limited potential financial gain from developing a new antibiotic. Antibiotics for many infections are typically used on a timescale of days-weeks, limiting their market potential. This is in contrast to drugs such as antihypertensives and antidiabetics which are typically used on a timescale of years (8). Scientific reasons include the problem of identifying good antibacterial targets which could give a suitable spectrum of activity. Furthermore, the low hit rate observed in screening campaigns against bacterial targets is problematic. This is illustrated by the experience from GlaxoSmithKline (GSK), which screened up to 530 000 compounds against 70 different targets over a period of seven years. This returned hits for 14 of the targets, with a total of five leads generated. All of these were subsequently dropped from further development (9). It has been speculated that a large part of the failures in screening for antibacterial compounds can be explained by the inadequate chemical libraries available. Most corporate and publicly available libraries are heavily biased towards types of compounds active against human drug targets. Furthermore, successful antibiotics in clinical use are characterized by significant differences in their properties compared to non-antibacterial drugs, including higher polarity, smaller size and larger proportion of covalent binders (10).
1.1.1 How can we combat antimicrobial resistance?

1.1.1.1 Pathogen identification, susceptibility testing and surveillance

A key component is pathogen identification and susceptibility testing of clinical isolates. This enables targeted therapy, and can help in guiding empirical treatment (treatment prior to definite identification of the pathogen and susceptibility testing) when combined with surveillance. The combination is also important for limiting the spread of resistant organisms and can help in directing the development of new antibacterials.

1.1.1.2 Prudent use of a limited resource

Antibiotics are definitely a limited resource given that the number of suitable drug targets in bacteria is relatively low. Furthermore, antibacterial resistance shows a clear correlation to the level of usage, both in humans (11–13) and animals (14). In many countries, antibiotics are available over the counter without prescriptions and are frequently being prescribed on inadequate indications (e.g. for viral infections).

Another important factor to consider is the antimicrobial spectrum of the antibiotics – ideally an antibiotic should only target the bacteria causing the infection at hand. This reduces the risk of resistance development as well as potential for side effects. However, since a large proportion of infections are treated empirically, agents targeting only a single bacterium are not always ideal. Typically, rapidly progressing, life threatening infections such as meningitis and severe sepsis require a broader spectrum of activity. On the other hand, slowly progressing and non-fatal infections can be treated with agents with a narrower spectrum, given that the causative agent is sensitive.

The use of antibiotics as growth promoters in animal feed is prohibited in the EU, but is commonly used elsewhere, including the US, despite minimal production gain (15). Even in countries where such a misuse is prohibited, the use of veterinary antibacterials for treatment and prophylaxis differs significantly. A European study showed that the consumption in ten different countries differed by a factor ten (mg antibiotic consumed / kg produced meat) between low consumption countries (Sweden, Norway and Finland) and high consumption countries (the Netherlands and France) (16). The potential for reduction of use should be obvious.

Release of antimicrobials as a result of manufacturing is another problem which has been investigated recently. Effluent from a sewage treatment plant in India, serving 90 different bulk drug manufacturers, was analyzed for a number of pharmaceuticals and high levels of many antibiotics were found. The levels of ciprofloxacin in the effluents were 28-31mg/L (17). These concentrations are 30 times higher than the susceptibility breakpoint for E. coli (R > 1mg/L) and roughly ten times higher than the maximal concentration attained in patient serum (Cmax < 3-4 mg/L) during therapy (18). Given that these effluents were released directly into nearby rivers (17), the risk for resistance development should be substantial.
In conclusion, a strict antibiotic policy should be implemented where antibiotics are only available by prescription, on adequate indications and with a spectrum of activity suitable for the indication. This has been advocated for the last 15 years by STRAMA (Swedish Strategic Programme for the Rational Use of Antimicrobial Agents and Surveillance of Resistance), which has shown that long term changes in antibiotic usage can be achieved, at least on a national level (19). Furthermore, veterinary use should be drastically reduced, given that animals are generally treated with the same classes of antibacterials as humans and that resistant bacteria can be transmitted between species (15).

1.1.1.3 Containment and prevention of dissemination

To avoid spread of resistant organisms within hospitals and to the society, adherence to basic hygiene routines (including protective clothing, alcohol based hand wash and adequate cleaning procedures) is important. The same goes for identification and isolation of infected or colonized patients (20). There is a strong correlation between a low MRSA prevalence and strict adherence to such procedures (21). Furthermore, there is an association between implementation of strict procedures and a decrease in the prevalence of MRSA (22).

Given that international travels are common nowadays, antimicrobial resistance is also spread between countries. In a study on healthy volunteers, with negative pre-travel samples, traveling from a low-prevalence setting (Sweden) to destinations outside of northern Europe, 24 % tested positive for colonization with extended spectrum β-lactamase (ESBL) producing bacteria upon return. Notably, 88% of travelers returning from India were ESBL positive (23). This is especially problematic given the recent isolation of Gram-negative bacteria expressing the NewDelhi metallo-β-lactamase 1 (blaNDM-1). These bacteria are generally resistant to most available antibiotics including penicillins, cephalosporins, carbapenems, fluoroquinolones, tetracyclines and aminoglycosides (24, 25).

1.1.1.4 Development of new antibacterials

The last part involves the development of new antibacterials, and at the heart of antibiotic development is the characterization of potential antibacterial drug targets. Although antibiotic development is a far from trivial task (7, 9, 10), it needs to be continued nevertheless. It has been suggested that new approaches, in the scientific, regulatory and commercial parts of the development, need to be adopted (26–28).

One way of rapidly accessing “new” antibiotics is to rediscover old drugs and drug candidates which, for various reasons, are not widely available or have not been registered. These reasons might include inadequate spectrum of activity, frequent side-effects, lower efficiency or unfavorable pharmacokinetics, all of which might be acceptable in a situation of increased resistance. In a recent European study, the accessibility of many older antibiotics such as fosfomycin and colistin, was found to be highly variable and with a large potential for improvement (29).
1.2 WHAT IS A GOOD DRUG TARGET?

It has been suggested that a good drug target is a target for which a drug with desirable properties exists. However, this is somewhat of an oversimplification which affords little help in the evaluation of new potential drug targets. For the sake of simplicity, only the properties of potential antibiotic drug targets will be considered here.

1.2.1.1 Essentiality

A key property is that the targeted function is essential for the bacterium of interest. Herein, essentiality is defined as the inability for the bacterium to live or proliferate in the absence of said function. Often, drug targets for antibiotics are enzymes that take part in biochemical pathways such as the synthesis of DNA, proteins or the cell wall. If multiple parallel pathways exist, combined targeting of the different isoenzymes is needed. Thus, a target should be essential in its own right or be very similar to the ones constituting the redundancy, allowing for parallel targeting (30).

In the literature, there seems to be a great deal of confusion and conflicting views about how essentiality of a gene should be defined. In the following scenarios I will consider a gene to be essential:

- If the gene can be disrupted or removed from the chromosome only with the presence of an extra copy of the gene. This can be supplied either on a plasmid or on another location on the chromosome. The extra copy can be placed under an inducible promoter, enabling verification that the obtained mutants are non-viable or unable to grow without added inducer.

- If the gene of interest is placed under an inducible promoter and the bacteria are only able to grow when inducer is added. Care should be taken to avoid potential polar effects.

- If the gene of interest can be disrupted or removed only in the presence of an additive supplementing for the deficiency introduced. Care should be taken to avoid potential polar effects on genes belonging to the same operon. Therefore, scarless methods or other methods allowing read-through into adjacent genes are preferred.

1.2.1.2 Absence, difference or non-essentiality in the host

If a function is not present in the host, this might be a good starting point for consideration as a drug target. An example of a function which is essential in bacteria but not present in human cells is the synthesis of the cell wall. This is probably a part of the reason why compounds interfering with the synthesis of cell wall represent the most common mechanism of action for antibiotics in clinical use as well as compounds in development (6). This is illustrated by the β-lactams which interferes with cross-linking of the cell wall.
Another possibility is that the function of the target is needed in host cells, albeit with a large difference between the host function and the bacterial counterpart, permitting selective targeting. This is illustrated by trimethoprim, which binds to bacterial dehydrofolate reductase with a 1000-fold higher affinity than the human counterpart.

Even if identical functions exist in bacteria and humans and the function as such is essential for both, this function could potentially be useful as an antibacterial target given that the host have additional systems acting as backup. This will be discussed in more detail later in relation to the thioredoxin and glutaredoxin systems.

1.2.1.3 Assayability

Let us assume that an essential gene, without any close homologue, in a hypothetical bacterium of considerable medical importance has been identified.

In order to develop a new class of antibiotics targeting the function of this gene, some kind of assay to detect interference is needed. Since antibacterial targets are often enzymes, compounds interfering with the catalytic conversion of a substrate to product (enzyme inhibitors) are typically sought after.

Initially, small or large collections of compounds are usually screened for the desired property. Ideally, this should be done in an assay which is cheap and easy to parallelize. The hits found in the primary screen usually need further optimization to generate one or more leads, which can progress into further development. Alternatively, compounds can be screened directly on bacteria for growth inhibition or some other property that can easily be assessed after which the target can be identified.

1.2.1.4 Auxiliary information

Generally, the more you know about the target of interest the better. Auxiliary information might for instance include structural information, which can help in rational optimization of inhibitors. Sometimes, a detailed protein structure can even be used for in silico (in the computer) screening of potential inhibitors, bypassing the need for large scale screening of compounds in the laboratory (31). Nonetheless, suggested inhibitors also need to be verified experimentally.
1.3 **HELICOBACTER PYLORI**

*Helicobacter pylori* is a Gram-negative, slow-growing, microaerophilic rod (32) which was isolated in 1983 from patients with chronic gastritis and duodenal and gastric ulcers by Barry Marshall and Robin Warren (33) who were awarded the 2005 Nobel Prize in Physiology or Medicine for their discoveries. Eventually it was accepted that peptic ulcer disease is essentially an infectious disease curable by antibiotics. Apart from its role in chronic gastritis and gastroduodenal ulcer disease, the bacterium has been recognized as a definite carcinogen due to the strong correlation to gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (34, 35).

Estimations suggest that half of the global population is infected, with a higher prevalence in developing countries than in industrialized ones (32, 36). In the Kalixanda Study, where 1001 randomly selected adults from two communities in northern Sweden were subjected to gastroscopy (37), 33.6% were culture positive for *H. pylori* (38) and 4.1% had ulcers with an equal distribution between gastric and duodenal localization (39).

How does a bacterium manage to colonize the highly inhospitable environment in the stomach? Given that the environment in the stomach is highly acidic (pH down to 1-2), an important virulence factor is urease, which converts urea to ammonia and carbon dioxide. The ammonia acts as a buffer, allowing the bacterium to survive short term exposure of low pH. Other virulence factors important for colonization is motility, which permits *H. pylori* to enter the mucous layer, adhesins permitting adhesion to epithelial cells and various toxins giving the bacterium access to nutrients (32, 34).

Colonization of the host by *H. pylori* usually results in a strong inflammatory response with a massive production of reactive oxygen species (40). It might seem somewhat surprising that the bacterium is able to withstand this oxidative onslaught and establish persistent infections, given its strict microaerophilic *in vitro* growth requirements. Some of the systems responsible for protection against oxidative stress are thioredoxin dependent (41).

The standard triple therapy consists of a proton pump inhibitor (PPI) and two out of three of the following antibiotics: clarithromycin, amoxicillin and metronidazole. On a Swedish note, antimicrobial resistance is rather uncommon as illustrated by the Kalixanda Study (38) and treatment results are still good. Internationally, the picture is very different. Increasing resistance has necessitated the development of more elaborate and costly therapies, resulting in increased side effects and reduced patient compliance (42–44). Perhaps even more problematic, is that these therapies tend to include antibiotics such as levofloxacin, moxifloxacin and rifabutin, which are normally reserved for other types of infections. The need for development of novel antimicrobials targeting this gastric pathogen should be obvious.
1.4 BACILLUS ANTHRACIS

Bacillus anthracis, the causative agent of anthrax, is a Gram-positive, spore-forming rod. The bacterium derives its name from the Greek word for coal which refers to the black, coal-like escars sometimes formed during the cutaneous form of infection (45). B. anthracis belongs to the Bacillus cereus group and the same genus as the nonpathogenic bacterium B. subtilis which has been used extensively as a model organism (46).

B. anthracis has two major virulence plasmids; pOX1 and pOX2. pOX1 encodes the tripartite toxin whereas pOX2 encodes genes responsible for synthesis of the antiphagocytic poly-D-ɣ-glutamic acid capsule. The tripartite toxin is composed of protective antigen (PA) in complex with either lethal factor (LF) or edema factor (EF). PA mediates cellular uptake, whereas LF and EF mediates toxicity. Loss of either plasmid results in dramatic attenuation of virulence (47).

Anthrax exists in three major forms; cutaneous, inhalational and gastrointestinal. In the cutaneous form, spores are introduced through the skin whereas in the inhalational form, they are inhaled. In the gastrointestinal form, spores or vegetative bacteria are ingested. Spores phagocytized by macrophages germinate intracellularly after which lysis occurs and bacteria escape to multiply extracellularly (48).

The cutaneous form represents about 95% of reported cases and it has been estimated that there are about 2000 global cases annually. The mortality rate has been estimated to be up to 20% for untreated infections and less than 1% with early, appropriate treatment (49). Mortality in inhalational anthrax has historically been close to 100%, but this can be lowered by early treatment with adequate antibiotics and supportive care. Out of the 11 cases found during the 2001 anthrax letter attacks, 45% died (50).

In 2000, a Norwegian heroin user succumbed to a fourth form of anthrax which was named injectional anthrax due to the route of administration (sc. injection) (51). In late 2009, Scotland experienced an epidemic of injectional anthrax with a total of 31 cases, out of which 11 died (52). Additional cases were identified in other parts of the UK and Germany (49). Although it was originally assumed that the heroin had been contaminated in Afghanistan, a recent molecular epidemiological investigation suggest that contamination along the trade route was more likely, given the high degree of similarity to Turkish isolates (53).

During the course of a infection, vegetative B. anthracis cells divide rapidly in the host and can reach densities of up to $10^7 - 10^8$ bacteria per milliliter blood (48). The rapid growth necessitates efficient systems for synthesis of deoxyribonucleotides required for replication and repair of DNA. Additionally, the need for systems protecting the bacterium against oxidative stress imposed by the host immune system should be obvious.
Naturally occurring strains of *B. anthracis* are generally sensitive to a number of commonly used antibiotics including fluoroquinolones, tetracyclines, penicillins and aminoglycosides (54). Current guidelines usually recommend the combination of a fluoroquinolone or doxycycline with 1-2 additional antibiotics as empirical treatment for symptomatic patients. In a situation of suspected mass-exposure, such as after intentional release in a terrorist attack, post exposure prophylaxis with either ciprofloxacin or doxycycline is recommended (55, 56).

Disturbingly, resistance to most relevant antibiotics can easily be selected for *in vitro* (57), which could prove to be disastrous in a scenario intentional release. *B. anthracis* is also naturally resistant to most cephalosporins (45), which is a cornerstone in the empirical treatment of serious infections such as pneumonia, sepsis and meningitis.
1.5 RIBONUCLEOTIDE REDUCTASE

Deoxyribonucleic acid (DNA) is the macromolecule responsible for maintaining the genetic information in all living cells. To be able to replicate and repair DNA, a balanced supply of the monomer building blocks, deoxyribonucleoside triphosphates (dNTPs), is required.

During the 1950s, Peter Reichard and colleagues made the observation that ribonucleosides were incorporated into both RNA and DNA (58), whereas deoxyribonucleosides could only be incorporated into DNA, leading to the conclusion that there was an enzymatic activity responsible for the conversion of ribonucleosides to deoxyribonucleosides (59). This eventually led to the discovery of ribonucleotide reductase (RNR) in extracts of E. coli (60, 61) which was subsequently found to contain a stable tyrosyl radical (62) localized to tyrosine 122 (63). Although this was the first protein radical to be discovered, radicals have been found in many different proteins since (64).

RNR catalyzes the conversion of ribonucleotides to deoxyribonucleotides as outlined schematically in Figure 1, and represents the only mechanism for de novo synthesis of deoxyribonucleotides making it essential in most organisms (65).

Figure 1. Conversion of ribonucleotides to deoxyribonucleotides. Ribonucleotide reductase (RNR) converts ribonucleotides to deoxyribonucleotides. The most common substrates are diphosphates (PP) which are then phosphorylated to triphosphates, whereas a few RNRs use triphosphates directly (PPP). The hydroxyl group (OH) which is exchanged for a hydrogen atom (H) is highlighted in bold. The base is adenine (A), thymidine (T), cytosine (C) or guanine (G).

The seemingly simple reaction requires a quite complicated catalytic mechanism involving the generation of a stable radical, a long range transfer of the radical and the supply of two electrons from an external electron donor (65). The enzymatic activity is subject to a complex allosteric regulation to ensure a balanced supply of dNTPs (66, 67).
1.5.1 Different classes of RNR

There are three major classes of RNR, which differ in their overall structure, metal-cofactor requirement, tolerance or requirement for oxygen and use of external electron donor (65). RNRdb (www.rnrdb.molbio.su.se) is a manually curated database, collecting information about ribonucleotide reductases in various organisms (68). This is a veritable treasure trove for RNR scientists. Below, some aspects of the different RNRs will be discussed with a focus on their role for proliferation and virulence in different bacteria.

1.5.1.1 Class I – aerobic

Class I enzymes contain two major subgroups (Ia and Ib) differing in their genomic organization, metal cofactor requirement and allosteric regulation of activity (65, 66). The recently proposed subgroup Ic (69), will not be discussed further in this thesis.

Common for the class is that the protein is made up of two different nonidentical subunits (NrdA (α) and NrdB(β) for Ia and NrdE (α) and NrdF(β) for Ib) typically found in an α2β2 configuration. The radical is generated on a tyrosine residue in the β subunit which is then transferred to a cysteine in the active site of the α subunit, giving rise to the catalytic cysteinyl radical. During the course of a catalytic cycle, a disulfide is generated in the active site. This disulfide is reduced by the C-terminal cysteines located on flexible tail, which swings into the active site. The C-terminal disulfide is in turn reduced by external electron donors which can be glutaredoxin, thioredoxin or NrdH redoxin depending on the type of RNR and species. Since the formation of the radical is oxygen dependent, type I RNRs are only active in the presence of oxygen (aerobic conditions) (65).

Type Ia RNRs, as exemplified by the E. coli enzyme, contain an iron cofactor in the β-subunit which is essential for radical generation. This particular type of RNR is usually the only RNR present in higher eukaryotes such as humans, but is also frequently found in bacteria capable of growing under anaerobic conditions together with class II and/ or class III enzymes (68).

The first type Ib RNR was identified in Salmonella typhimurium as a gene able to complement a temperature sensitive mutant of the type Ia RNR in E. coli (70). The protein was recombinantly expressed in E. coli and was characterized as having an iron cofactor and the ability to use glutaredoxin but not thioredoxin as electron donor (71). However, a growing body of evidence suggests that type Ib RNRs instead use a manganese cofactor (72–76), where NrdI, a small flavodoxin-like protein, is needed for radical generation (77–79). Some of this development has recently been summarized (80). Interestingly, type Ib enzymes appears to be completely absent from the genomes of eukaryotes (including humans), but are common in bacteria, most of which do not encode other aerobic RNRs (68). This suggests that type Ib RNRs could be viable drug targets given that inhibitors targeting type Ib but not Ia RNRs can be found.
1.5.1.2 Class II – aeroboindifferent

Class II RNRS neither requires nor is inhibited by oxygen and can thus be classified as indifferent to oxygen, or aeroboindifferent. They contain a single α-subunit (NrdJ) and generate the cysteinyl radical with the help of adenosylcobalamin instead of a separate β-subunit. Thioredoxins or glutaredoxins are used as external electron donors (66). Class II enzymes predominantly exist in bacteria, some of which also contain class I and/or class III enzymes (68).

1.5.1.3 Class III – anaerobic

Class III RNRS are only active under anaerobic (oxygen free) conditions. Two different subunits are required for activity; the large catalytic subunit (NrdD) and the small radical generating unit (NrdG). NrdG contains a redox active iron-sulfur cluster, which together with S-adenosyl methionine (SAM) and reduced flavodoxin generates the radical. The radical is sensitive to oxygen, thus explaining the protein's general sensitivity to oxygen (66). The external electron donor for class III enzymes is formate (81). Class III RNRS are most often found in bacteria and are absent from most eukaryotes including humans. In bacteria, class III enzymes are found both in strict and facultative anaerobes. In the latter, they are combined with class I and/or class II enzymes (68).

1.5.2 A closer look at RNR in selected bacteria

Some bacteria deserve a closer examination of their RNR with a focus on essentiality and role in virulence. E. coli does so, on the basis of its well characterized history and role as reference organism, whereas the other bacteria are in more direct focus of this thesis.

1.5.2.1 Escherichia coli

Most strains of E. coli with available sequence information contains one Ia, one Ib and one class III RNR (68). The presence of both aerobically and anaerobically active RNRSs is expected given that the bacterium is a facultative anaerobe. However, the presence of both a class Ia and a Ib RNR is a bit more surprising.

Despite the presence of two aerobic RNRSs in E. coli, the type Ia is essential under aerobic conditions unless the type Ib is overexpressed (82–84). In a recent study, E. coli could survive solely on its type Ib RNR when subjected to iron starvation, H\textsubscript{2}O\textsubscript{2}-stress or supplementation of the growth medium with high levels of manganese (75). This might indicate a role for the type Ib RNR under iron-deprived conditions such as during an infection. To limit the access to iron for the microbe is an important defense strategy for the host.
In a series of experiments, it was shown that both of the subunits of class III RNR in *E. coli* are essential for growth under strict aerobic conditions. However, even trace amounts of oxygen was enough to promote growth by the class I enzymes (85). This was later verified (84).

**1.5.2.2 Helicobacter pylori and Campylobacter jejuni**

*H. pylori* contains only a single type Ia RNR (86), which might explain its inability to grow under anaerobic conditions. So far, no experimental data has been published regarding RNR from this gastric pathogen.

The closely related microaerophile *Campylobacter jejuni*, which causes colitis and diarrhea, also encodes a single Ia RNR (87) and is unable to grow under strict anaerobic conditions. In a recent study, the authors showed that *C. jejuni* is able to respire without oxygen as the terminal electron acceptor. On the other hand, DNA synthesis was inhibited. Anaerobic conditions resulted in filamentation, a morphology also seen when bacterial cultures were treated with hydroxyurea – a well known inhibitor of ribonucleotide reductases (88).

Whether or not RNR in these bacteria could be viable drug targets remains to be established. This is far from obvious given that humans also rely on type Ia RNRs for synthesis of deoxyribonucleotides. However, one could imagine inhibitors selective for the bacterial enzymes or compounds which are not absorbed from the gastrointestinal tract, thus giving low systemic exposure.

**1.5.2.3 Bacillus anthracis, cereus and subtilis**

*B. anthracis*, *B. cereus* and *B. subtilis* all have type Ib RNRs but not type Ia RNRs (68). On a general note *B. subtilis* seems to be missing anaerobic RNRs whereas most *B. cereus* and some of *B. anthracis* strains appear to have genes encoding for type III RNRs.

Both subunits of the type Ib RNR are essential for growth of *B. subtilis* under aerobic conditions on rich media (89). To the best of my knowledge, essentiality of the corresponding genes in *B. cereus* and *B. anthracis* has not been investigated. However, it seems likely that they are essential under aerobic conditions.

Whether or not *B. subtilis* is able to grow under strict anaerobic conditions without exogenously added deoxyribonucleotides, is still under debate (90, 91).

The iron form of the type Ib RNR from *B. anthracis* has been studied as a potential drug target. Compounds showing a selective inhibition over type Ia RNRs *in vitro* were identified (92, 93). Certain compounds showed some activity against *B. anthracis* and a moderate selectivity relative to *E. coli* (92).
1.5.2.4 Staphylococcus aureus

*S. aureus* is a Gram-positive cocci, capable of growing both aerobically and anaerobically. It is an important human pathogen causing a wide variety of clinical conditions including endocarditis, septicemia as well as skin and soft tissue infections (94).

*S. aureus* encodes one class Ib and one class III RNR. The class III RNR, which is essential for anaerobic growth of *S. aureus* (95), was shown to be highly important for virulence in a mouse model of septic arthritis, and for the ability of the bacterium to persist in the kidney parenchyma after an intravenous challenge (96).

Although essentiality of the type Ib RNR for aerobic growth of *S. aureus* has not been formally shown, it is likely, given the results from a transposon based study, which suggest that both NrdE and NrdF are essential (97). In another study, the type Ib enzyme was characterized in its pure form and was found to be active (98). The low activity found could likely be explained by the use of an iron-loaded form of NrdF.
1.6 THE THIOREDOXIN AND GLUTAREDOXIN SYSTEMS

Many organisms, including *E. coli* and man, have two major protein disulfide reductase systems – the thioredoxin and the glutaredoxin system (*Figure 2*). The thioredoxin system is composed of NADPH, thioredoxin reductase (TR) and thioredoxin (Trx) shuttling electrons to the terminal substrates (99). When present, NrdH can also be considered a part of the thioredoxin system as discussed later in the introduction. The glutaredoxin system on the other hand consists of NADPH, glutathione reductase (GR), glutathione (GSH) and glutaredoxin (Grx) (100).

![Flow of electrons](image)

*Figure 2. The thioredoxin and glutaredoxin system.*

1.6.1 Thioredoxin

Thioredoxin was originally discovered in *E. coli* as a hydrogen donor for ribonucleotide reductase (RNR) (101). Four years later, the amino acid sequence was elucidated (102) and in 1975, the first high-resolution structure was presented (103).

Thioredoxins are small (approx. 12 kDa), heat stable proteins with a well characterized fold. The thioredoxin fold is composed of a central β-sheet surrounded by α-helices. Although the minimal Trx fold requires four β-strands and three α-helices (104), thioredoxin as exemplified by *E. coli* Trx1 contain a five-membered β-sheet surrounded by four α-helices (103). The thioredoxin fold is present in a wide variety of proteins with different functions including glutaredoxins, NrdH redoxins, protein disulfide isomerases and glutathione S-transferases (104). Thioredoxins typically have WCGPC active sites, but exceptions do exist as exemplified by Trx2 from *H. pylori* which has a WCPDC active site (105) and by Trx2 from *B. anthracis* as discussed in paper II (106).
In addition to its role as an electron donor for RNR, thioredoxin also acts as a general protein disulfide reductase with an important role in upholding of the reducing intracellular milieu and protection against oxidative stress (107–109). Specific disulfide targets include methionine sulfoxide reductase (MSR), 3'-phosphoadenylylsulfate (PAPS) reductase (110), and thioredoxin dependent peroxiredoxins (111, 112).

PAPS reductase catalyses the conversion of 3'-phosphoadenylylsulfate (PAPS) to sulfite and is required for sulfur assimilation from sulfate. PAPS reductase is generally essential in bacteria unless a reduced sulfur source such as methionine, cysteine or glutathione is present. MSR on the other hand reduces methionine sulfoxides in proteins and is important for protection against oxidative stress (113). The same goes for the peroxiredoxins which scavenge organic and inorganic peroxides (114).

Thioredoxin also has a number of functions which does not involve the transfer of electrons to a substrate, but rather where it acts as a structural component. The most well-known of these is perhaps *E. coli* thioredoxin and its role as a subunit of the phage T7 DNA polymerase, for which it required for activity (115–117).

### 1.6.2 NrdH

NrdH was identified as an electron donor for the type 1b RNR from *Lactococcus lactis* with a CMQC active site (118). Further characterization of the *E. coli* counterpart, with a CVQC active site, revealed a thioredoxin activity profile despite the sequence similarity to glutaredoxins. It was speculated that this might result from the inability to bind GSH (119) which was later confirmed by the crystal structure that showed the absence of a GSH binding site in NrdH. Furthermore, the structure suggested a putative TR binding patch, explaining the ability for TR dependent reduction (120).

### 1.6.3 Thioredoxin reductase

Thioredoxin reductases are widely distributed, FAD containing enzymes which catalyze the NADPH-dependent reduction of thioredoxins and NrdH-redoxins (121). Thioredoxin reductase was discovered in, and partially purified from, *E. coli* in parallel with the work on thioredoxin (122). It was further purified and characterized a few years later (123).

The most well studied TR is *E. coli* enzyme, for which crystal structures have been determined of several different forms. This includes the C138S in oxidized, FO form (124), wild type in oxidized FO form (125) and fully reduced FO form (126). Finally, the structure of a complex of *E. coli* TR C135S trapped in a complex with an active site mutant (C35S) of *E. coli* Trx1 was published in 2000 (127).
Whereas thioredoxins from most species show a high degree of structural and functional similarity, this is not the case for their respective reductases. Two major groups have evolved. The low molecular weight group is exemplified by the bacterial enzymes and the high molecular weight group is exemplified by the mammalian enzymes (121).

1.6.3.1 Bacterial thioredoxin reductases

The bacterial enzymes have a subunit weight of 35 kDa and utilize a CAT/VC active-site. The active site is positioned in a crevice in the central parts of the molecule, and the enzyme needs to undergo a 67 degree domain rotation during their catalytic cycle alternating between the FO and the FR forms (127). The flow of electrons is from NADPH via FAD to the active-site disulfide (121). The substrate specificity of the bacterial thioredoxin reductase group is generally quite narrow and is largely confined to its respective thioredoxins, and when present, NrdH (119).

1.6.3.2 Mammalian thioredoxin reductases

The mammalian enzymes on the other hand, have a subunit weight of about 55 kDa and show a high degree of structural similarity to glutathione reductases (128). They utilize a GCUG active-site, where U is the unusual and highly reactive amino acid selenocysteine, positioned at a flexible C-terminal extension (129). The flow of electrons is from NADPH via the FAD and the N-terminal redox-center to the selenosulfide which then reduces the substrate (130). The mammalian enzymes have a much broader substrate specificity which includes, apart from thioredoxins from different species, various low molecular weight compounds such as hydrogen peroxide, selenite and DTNB (131). Mammals, including humans, have two major isoforms of TR, TR1 which is cytosolic and TR2 which is mitochondrial.

Thioredoxin reductase has been suggested to be a drug target for certain anticancer drugs such as cis-platin and arsenic trioxide acting through alkylation of the highly reactive selenocysteine. Since this falls outside of the scope of this thesis, the interested reader is encouraged to seek further information in the following reviews (132, 133).

Interestingly, although null mutations for TR1 are embryonically lethal in mice (134, 135), the gene is dispensable later if glutathione is present. This was demonstrated by conditional knockouts which allowed for normal growth and proliferation of hepatocytes – even under conditions of rapid growth such as in the re-growth phase after a partial hepatectomy (136, 137). This raises questions about the validity of TR1 as a potential drug target in cancer therapies, although a toxic gain of function mechanism or combination with other drugs targeting glutathione dependent pathways could be viable.
1.6.4 Glutaredoxin

Given the role of thioredoxin as an electron donor for ribonucleotide reductase, it was surprising that mutants of phage T7-negative *E. coli* (115), without detectable thioredoxin activity (117, 138), were viable, exhibited an almost normal phenotype and had the ability to synthesize DNA. In 1976, this led to the discovery of another protein which was able to act as an electron donor for RNR. Since the activity was dependent on glutathione, it was named glutaredoxin (138) which was subsequently purified to homogeneity (139) and further characterized (140).

Glutaredoxins share the same basic architecture with thioredoxins (the thioredoxin fold), although they are typically smaller with four β-strands, three α-helices and a molecular weight of approximately 10 kDa. Another class of glutaredoxins, as exemplified by *E. coli* Grx2, is characterized by a molecular weight of approximately 24 kDa and an overall fold similar to that of glutathione-S-transferases (141). The most commonly found active site amongst glutaredoxins is the dithiol motif CPYC (142). However, monothiol glutaredoxins do exist as exemplified by *E. coli* Grx4 which encodes a CGFS active site (143, 144).

In organisms, like *E. coli* and humans, which have both the thioredoxin and glutaredoxin system present, some functions overlap whereas other functions are distinct for the glutaredoxin system. The former is exemplified by the reduction of ribonucleotide reductase and PAPS reductase, whereas the latter is exemplified by glutaredoxins role in reduction of glutathionylated proteins (142).

1.6.5 Glutathione and glutathione reductase

Glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is a tri-peptide composed of glutamic acid, cysteine and glycine. GSH is synthesized by the enzymes γ-glutamylcysteinyl synthetase (GshA) and glutathione synthetase (GshB) in a two step process requiring ATP. Apart from its role in the reduction of glutaredoxins, GSH has a wide variety of functions including direct neutralization of reactive oxygen species (ROS) and detoxification of xenobiots (145). Glutathione reductase (GR) is the FAD containing enzyme responsible for regeneration of glutathione (GSH) from the oxidized form (GSSG) at the expense of NADPH (146).

1.6.6 The thioredoxin and glutaredoxin systems in *E. coli*

Combined null mutants of Grx1 and Trx1 were not viable unless the growth medium was supplemented with cysteine. However, they had a capacity for RNR reduction which led to the conclusion that either Trx1 or Grx1 is required for reduction of PAPS reductase, but that an alternative electron donor for RNR exists (147).
An *E. coli* strain with null mutations for Trx1 and Grx1 (148), was used to isolate two additional glutaredoxins (Grx2 and Grx3). Grx2 was unable to reduce *E. coli* RNR, whereas Grx3 showed an approximate catalytic efficiency of 2% relative to Grx1 (149). Given the low capacity of Grx3 for RNR reduction, the physiological role was not immediately clear.

Eventually, it was established that *E. coli* had at least one more redoxin capable of TR dependent reduction of disulfides (150). Trx2 was identified and shown to be capable of reducing insulin and the type Ia RNR but not the type Ib (151).

A fourth glutaredoxin (Grx4), with a monothiol CGFS active site was discovered in *E. coli*. Although likely essential, the exact physiological role is currently a bit unclear (143), given that it cannot replace Trx1, Trx2 or Grx1 in their respective roles in reduction of RNR and PAPS reductase.

All in all, *E. coli* has two thioredoxins and four glutaredoxins and requires either a functional thioredoxin or glutaredoxin system to sustain aerobic growth and produce dNTPs *in vivo*. The thioredoxin can be either Trx1 or Trx2, whereas Grx1 becomes essential in the absence of a functional thioredoxin sytems (84, 150).

### 1.6.7 Some bacteria do not produce GSH

Many bacteria do not use GSH – some of which are medically important pathogens such as the *Staphylococci* (*S. aureus* and the coagulase negative staphylococci including *S. epidermidis*) and *Mycobacteria* (including *M. tuberculosis*). This also holds true for the *B. cereus* group (including *B. anthracis*) and the common model organism *B. subtilis* (152–154).

*H. pylori* lacks the genes required for synthesis and regeneration of glutathione (GR) (86), but instead degrades GSH from the host using the extracellular enzyme γ-glutamyl transpeptidase (GGT) giving the bacterium access to glutamate (155). GGT results in the induction of oxidative stress and is required for colonization of the gastric mucosa in animal models (156). *H. pylori* isolates from patients with active peptic ulcer disease have a significantly higher GGT-activity compared to isolates from patients without ulcers (157).

The absence of glutathione should make these bacteria heavily dependent on the thioredoxin system for reduction of ribonucleotide reductase and protection against oxidative stress.
1.7 THIOREDOXIN SYSTEMS IN BACTERIA DEVOID OF GSH

1.7.1.1 *Helicobacter pylori*

In the sequenced genome of *H. pylori* 26695, there are two genes annotated as thioredoxins and two genes annotated as thioredoxin reductases (86). Windle *et al.*, isolated one thioredoxin (Trx1) and one thioredoxin reductase from its native source, verified their activity and observed that Trx1 is accumulated under conditions of stress induced by incubation with anti-*H.pylori* antibodies (158). Another study characterized Trx1 and Trx2 as substrates for TR and electron donors for alkyl hydroperoxide reductase (AhpC). Interestingly, the catalytic efficiency of Trx1 as a substrate for TR was approximately three-fold lower, predominantly due to a six-fold higher $K_m$, relative to the *E. coli* counterpart. Trx2, with its atypical CPDC active-site, showed similar catalytic properties with model disulfides, but was unable to reduce AphC (105).

Surprisingly, a combined null-mutant for both Trx1 and Trx2 in *H. pylori* was viable. However, it could only be isolated in an atmosphere with very low oxygen tension (2%), grew slowly to a low final density and was very sensitive to various forms of oxidative and nitrosative stress (159). This might indicate the existence of an alternative pathway for reduction of RNR and other protein disulfides.

The annotation of a second gene as a thioredoxin reductase, based on its sequence similarity to TR from other species (86) opened up the possibility of further redundancy in the system. However, the gene (HP1164) does not encode a protein with a potential CXXC active site. The protein was later shown, rather than acting as a TR, to be a combined flavodoxin and quinone reductase and should be reannotated accordingly (160). Thus, *H. pylori* appears to have only one TR.

Although essentiality for the thioredoxin system has not been shown *in vitro*, this is likely the case *in vivo*, given the very fragile phenotype for the combined Trx1 and Trx2 null-mutant.

1.7.1.2 *Bacillus anthracis, cereus and subtilis*

In *B. subtilis*, Trx and TR are both essential for growth on rich laboratory media (89, 161). This essentiality could be overcome by the supplementation of the growth medium with deoxyribonucleotides and one of the two sulfur containing amino acids, cysteine or methionine. However, the growth was still severely retarded, the sporulation efficiency was decreased by more than five orders of magnitude and the Cytochrome c oxidase activity decreased by more than 99% (162).

Under certain conditions, conditional null mutants of Trx in *B. subtilis* which are not dependent on added inducer can be obtained. However, this can be attributed to the general leakiness of the promoter and the accumulation of suppressor mutations, allowing for read-through into the gene (162–164).
In *B. cereus*, very little has been published with regards to the thioredoxin systems. There is one article describing that *B. cereus* NrdH can function as an electron donor for the type Ib RNR of both *B. cereus* and *B. anthracis* (79). As for the situation in *B. anthracis*, to the best of our knowledge, nothing had been published previously. This was addressed in paper II (106) as will be discussed in more detail later.

### 1.7.1.3 *Staphylococcus aureus*

Aharonowitz and colleagues studied the thioredoxin system from *S. aureus* and observed that both Trx and TR were upregulated on a transcriptional level when exposed to oxidative stress. Furthermore, they were unable to isolate null mutants of the gene encoding TR, suggesting that it is essential (165). This was later corroborated by another study which utilized transposome based techniques to find essential genes. This study suggested that both Trx and TR are essential (97). Although none of these studies fulfill the formal criteria for designating a gene as essential (as discussed in section 1.2), they are strongly suggestive of essentiality.

In a later study, an NrdH-redoxin was identified which was not part of the usual NrdHIEF operon. The active site of the protein was CPPC instead of the CVQC sequence found in *E. coli* NrdH. The protein was active as a general disulfide reductase and could transfer electrons to the type Ib RNR, albeit with a low activity. The gene encoding NrdH was found to be non-essential and disruption of it was not associated with any obvious phenotype (98).

### 1.7.1.4 *Bacteroides fragilis*

*B. fragilis* is an aerotolerant, Gram-negative anaerobe. It is the most commonly isolated anaerobe in clinical samples and causes septicemia and abscesses. Important for its pathogenic potential is likely its relatively high tolerance for oxygen compared to other anaerobes.

Recently, TR was shown to be a key component for oxygen tolerance and for protection against oxidative stress in *B. fragilis*. Furthermore, a strain in which the gene encoding TR had been deleted, was severely attenuated and unable to form intraperitoneal abscesses in a mouse model (166). On a genomic level, the bacterium seems to have six different thioredoxins, out of which one is essential. Some of the other, nonessential thioredoxins, appear to be important for protection against oxidative stress (167). So far, no data on the pure proteins has been published. This could help verifying which of the six are actual thioredoxins and which might have other functions in the cell.
1.8 BACILLITHIOL – AN ALTERNATIVE TO GSH?

Some bacteria, including *B. anthracis*, *B. subtilis* and *S. aureus* produce the recently described low molecular weight thiol, bacillithiol (BSH) which is present at approximately 30-fold lower concentrations than GSH in *E. coli* (154).

The role of BSH is however, still a bit unclear given that strains with gene knock-outs abolishing synthesis of BSH in both *B. subtilis* (168) and *B. anthracis* (169) are viable, without obvious growth defects. Strains unable to produce BSH do however exhibit decreased sporulation efficiency, increased sensitivity to extremes of pH and ionic strength as well as increased sensitivity to the antibiotic fosfomycin (168, 169).

This does not paint the picture of attractive drug-targets for antibiotic development. Given the essentiality of TR and Trx1 in *B. subtilis* (89, 161), and the highly likely essentiality of TR and Trx in *S. aureus* (97, 165) a role for BSH as a substitute for GSH seems somewhat unlikely.
2 AIMS OF THE THESIS

The overall goals of this thesis were to characterize bacterial thioredoxin systems as potential drug targets and to develop inhibitors thereof. Since the thioredoxin system is generally dispensable in bacteria which produce glutathione, the focus was on bacterial species devoid of glutathione. A special emphasis was given to *H. pylori*, the causative agent behind gastroduodenal ulcers, gastric cancer and type B gastritis, as a representative for slow growing, chronic pathogens and *B. anthracis*, the causative agent of anthrax, for rapidly growing bacteria causing fulminant disease. Part of the thesis project was also devoted to methodological development in the area of cloning and recombinant protein expression. Some of the specific aims are listed below with the roman numerals denoting in which paper they have been addressed:

- To gain new structural insight on bacterial thioredoxin reductases in general, and the one from *H. pylori* in particular (I).

- To characterize the thioredoxin systems from *B. anthracis* as general protein disulfide reductases and electron donors for the essential protein ribonucleotide reductase. Furthermore, to sort out the apparent, several fold, redundancy suggested by the annotations of the genome (II).

- To characterize ebselen as an inhibitor of bacterial thioredoxin reductases on a biochemical and bacteriological level (III, IV).

- To establish a structure-activity-relationship for ebselen and derivatives thereof as inhibitors of bacterial thioredoxin reductases and as antibacterial compounds (IV).

- To develop a cloning methodology, retaining the flexibility of the Restriction Free (RF) cloning method while improving speed, simplicity and cost-effectiveness (II).
3 METHODOLOGY

Most projects require a number of different techniques, some of which are laborative in their nature and some of which are computer based. A few of the methods where some kind of development was undertaken are discussed below.

3.1 GENE CLONING

In this thesis gene cloning, or simply cloning, specifically relates to the process in which a copy of a gene is transferred from the genome of an organism into another vector such as a plasmid. A plasmid is a short, circular piece of DNA which is replicated independently from the chromosomal DNA and is commonly present in many bacteria. A plasmid used for this purpose typically encodes an origin of replication (ORI) where replication starts, integration sites for the desired gene and an antibiotic resistance marker which allows the host to survive under selective pressure of the specific antibiotic. Plasmids intended for recombinant protein expression also encodes a promoter region which allows the RNA polymerase to bind and initiate transcription, where the T7-polymerase system is popular (170, 171).

A typical traditional approach to cloning involves the PCR-amplification of the gene of interest, gel-purification of the amplified fragment, digestion with restriction endonucleases of the fragment and vector, annealing, ligation and transformation into recipient bacteria. This approach suffers from limited flexibility, given that specific restriction sites needs to be considered, and is inherently labour intensive.

In the era of structural genomics, the problems of parallelization associated with the traditional restriction-ligation cloning resulted in the development of Ligation Independent Cloning (LIC-cloning) which lends itself to parallel cloning of multiple targets. This method does however require special adaptation of the vectors (172) limiting the flexibility.

Another method, which addresses the limitations with regards to flexibility, is Restriction Free (RF) cloning which was originally developed by Geiser and co-workers (173) and further developed by van den Ent and co-workers who gave the method its name (174). The method is composed of an initial PCR, where the gene of interest is amplified by forward and reverse primers which are flanked by roughly 25 base pairs complementary to the destination vector. After gel purification, the PCR-product is used as primers in a linear amplification step, where the fragment is inserted in the destination plasmid, after which the mixture is treated with DpnI to digest the parental plasmid. The product is then used to transform E. coli. Although flexible, the method is still rather labour intensive, time consuming and does poorly lend itself to parallelization.

These limitations prompted us to develop ERF-cloning (Exonuclease dependent Restriction Free cloning), which is discussed in more detail in paper II (106).
3.2 RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

A prerequisite for many comprehensive biochemical investigations is pure protein in adequate amounts (usually mg-quantities). Traditionally this has been achieved by isolation of the protein from its native source. This is typically both time-consuming and labour intensive giving a low final yield, unless the protein of interest is highly abundant and/or has special properties allowing for simplified purification procedures.

With the development in molecular biology (especially in the area of gene cloning and with the ever-growing availability of sequenced genomes), recombinant protein expression in E. coli has largely taken over as the primary source for proteins for biochemical studies. Most often, E. coli harboring the gene of interest on a plasmid is grown to mid-log phase at 37°C, in a medium containing nutrients in the form of yeast extract and digested protein extracts, after which an inducer is added to the culture. This inducer, which is typically IPTG (Isopropyl β-D-1-thiogalactopyranoside) as for the T7-system, induces expression of the gene of interest (171). Expression of target proteins with IPTG often results in high expression of target protein, but can result in the formation of insoluble protein aggregates. Furthermore, the massive induction of expression often limits the final density of the bacterial culture to an OD$_{600}$ of approximately 2-3, thus lowering the final yield. These problems are especially prominent when protein production is done at 37°C, but can sometimes be partially circumvented by lowering the cultivation temperature, resulting in slower growth rates. Some structural genomics projects utilize a combination of rich, buffered mediums with expression of proteins at low temperature and the use of specialized equipment ensuring maximal aeration. This results in increased final cell densities (up to about OD$_{600} = 20$) and protein levels (172, 175).

Previous investigators have observed that high level production of the desired protein is sometimes induced without any exogenously added inducer (176, 177). However, the mechanism behind this was not fully understood.

In a seminal paper from 2005, FW Studier dissected the mechanism and found that the unintended induction was due to residual lactose from the casein hydrolysate which is converted to allo-lactose, in turn driving expression of the gene. This led to the development of auto-induction, in which a low level of glucose prevents uptake of lactose and permits growth to high densities. When glucose is exhausted, lactose which is also included in the medium is taken up and the protein of interest is produced. This allowed for high-density cultivation (final OD$_{600}$ up to ≥ 20) without the need constant observation of growth or specialized equipment such as fermentors (178). The method was further developed by BG Fox and co-workers (179, 180).
When a protein is recombinantly expressed in *E. coli*, intracellular expression is usually opted for, which means that the protein has to be extracted before purification. This can be achieved through a number of methods including physical methods such as sonication (181), detergents which dissolve the membrane (182), enzymes degrading the cell wall (183), compounds interfering with the integrity of the LPS layer (such as EDTA) and freeze-thaw treatments which disrupts the inner membrane through the formation of ice crystals (184). Often, methods need to be combined for efficient lysis.

In paper II (106), we used auto-induction for expression of the target proteins. By combining an optimized auto-induction medium with low temperature cultivation, we could obtain up to 800 mg of pure protein / litre bacterial culture. Furthermore, by including arabinose in the medium, and through the use of the bacterial strain XJB(DE3)Autolysis (ZymoResearch), which carries a chromosomal copy of λ-phage lysozyme under control of the pBAD promoter, we could easily lyse the cells by simply resuspending the bacteria in a hypotonic buffer followed by a freeze-thaw treatment. This allowed for facile processing of multiple constructs in parallel. When combined with nucleases to degrade DNA and RNA, it resulted in low-viscosity lysates, suitable for downstream processing.

A problem often encountered when FAD-containing proteins are over-expressed to very high levels, is that the cellular machinery for synthesis of FAD cannot keep up. It has previously been counteracted by including FAD in the lysis buffer, increasing the amount of holo enzyme (176). This strategy was adopted in both paper I (185) and II (106). In paper II, it resulted in a seven-fold increase in holo enzyme, compared to the unsupplemented control, as determined by the ratio of FAD absorbance to protein concentration (determined by the method of Lowry).
4 RESULTS AND DISCUSSION

4.1 PAPER I

*Helicobacter pylori* is heavily dependent upon the thioredoxin system for growth and defence against oxidative stress (159). The catalytic properties for the TR-TrxI couple is also quite different compared to the *E. coli* equivalent. When *H. pylori* TrxI was used as a substrate for its TR, $K_m$ was found to be six-fold higher (105) than for the *E. coli* couple. At the time, crystal structures of thioredoxin reductases from two different bacteria were available; several structures of the *E. coli* enzyme (124–127), and one of the enzyme from *M. tuberculosis* (186). Based on this, we wanted to investigate the thioredoxin reductase from this gastric pathogen from a structural point of view, with the hope of obtaining information that could be of value in drug development.

Crystals were rapidly obtained in the initial screens for the enzyme without added NADP$^+$. However, despite extensive optimization, crystals of this form diffracted to 3-4Å at best and were not pursued further.

When instead enzyme mixed with NADP$^+$ was used, crystals growing as thin plates appeared in the initial screens. Unfortunately, these crystals were very hard to obtain reproducibly and diffracted to a maximum of 2.8Å. Through the use of a combination of additives, detergents and streak-seeding, we were able to obtain reproducibly growing crystals which still grew as plates. After a couple of rounds of streak-seeding, crystals growing as thick, bright yellow rods (*Figure 3*) were obtained. These crystals reproducibly diffracted to better than 2Å resolution.

*Figure 3. Helicobacter pylori* thioredoxin reductase crystallized in the presence of NADP$^+$. 
We were able to determine the crystal structures of the oxidized and reduced forms of the enzyme at 1.7 and 1.45 Å resolutions, respectively. At the time, this represented the most highly resolved crystal structures of a bacterial thioredoxin reductase.

The overall architecture of the enzyme (Figure 4) was quite similar to the one from *E. coli* (124–127) and *M. tuberculosis* (186). However, there are distinct differences, especially in the thioredoxin binding area, which is less negatively charged in the *H. pylori* enzyme compared to the *E. coli* counterpart. This may potentially explain species-specific recognition of Trx by TR.

Figure 4. *Helicobacter pylori* thioredoxin reductase. Ribbon representation of the dimer of reduced *H. pylori* thioredoxin reductase (PDB code: 2Q0L). Bound FAD and NADP⁺ are shown in stick representation. Subunits are coloured by domain organization, with the FAD-binding domains in blue and the NADPH-binding domains in yellow (Picture courtesy of Dr. Edvard Wigren).

The structural information could potentially be used for rational design and optimization of inhibitors, but has hitherto predominantly been used as a reference for modeling and structure determinations (187–193).
4.2 PAPER II

To broaden the scope of the thesis, we decided to take a closer look at the thioredoxin systems from \textit{B. anthracis}, the causative agent of anthrax. Although neither \textit{H. pylori} nor \textit{B. anthracis} produce glutathione, they are quite different in a number of other aspects as discussed in some detail the introduction. When this project was started, there was no information available on the thioredoxin systems from \textit{B. anthracis} or the closely related \textit{B. cereus}. At the time of publication, there was one article demonstrating that \textit{B. cereus} NrdH together with TR could reduce RNR from \textit{B. anthracis} (79). Since the focus of this article was primarily on the metal content and radical in RNR, other possible combinations were not taken into account.

Our goal was to try to identify all possible thioredoxin reductases, thioredoxins and NrdH-redoxins in \textit{B. anthracis} and to characterize the flow of electrons from NADPH to the terminal substrates with an emphasis on ribonucleotide reductase.

Initial searches showed that a large number of genes were annotated as potential thioredoxin reductases, thioredoxins and NrdH-redoxins in \textit{B. anthracis} and to characterize the flow of electrons from NADPH to the terminal substrates with an emphasis on ribonucleotide reductase.

Results primary search
4 potential thioredoxin reductases
6 potential thioredoxins
3 potential NrdH-redoxins/grx-like

Experimental set
2 potential TR
5 potential Trx
3 potential NrdH/grx-like

Figure 5. Overview of the bioinformatic strategy. Annotated genes from the genome of \textit{B. anthracis Ames} (194) were accessed through the RefSeq database (195). Retrieved genes were manually inspected for a potential active site (CXXC) and genes were excluded when such a sequences was absent. Subcellular localizations were predicted using PSORTb and genes with a predicted extracellular localization were excluded. The rest of the genes were included in the experimental set and were cloned, expressed, purified and characterized.
For cloning of the genes, we developed a new cloning methodology which we named ERF-cloning. This cloning method retains the flexibility of restriction free cloning (173, 174) while significantly improving speed, ease of parallelization and cost effectiveness. This method could be useful in low-medium throughput laboratories where flexibility combined with speed should be of greatest interest. In high throughput laboratories, the ease of parallelization might be more important.

When we started characterizing the proteins, it quickly became apparent that there were few active players. Out of the two possible thioredoxin reductases, only TR1 was active. Potential TR2 was inactive in combination with all six potential redoxins in both the assays and was not reducible by NADPH or NADH. Out of the three potential thioredoxins, two were active. Trx1 could reduce both the low molecular weight disulfide DTNB and insulin which serves as a model for general protein disulfides. Trx2 on the other hand, could only reduce DTNB. This contrasts to the situation in H. pylori, where Trx2 can reduce both DTNB and insulin (105), despite an identical CPDC active site. Out of the three potential NrdH-redoxins, only one was active. When the different redoxins were characterized in more detail as substrates for TR, Trx1 was found to be superior, giving three-fold and 20-fold higher catalytic efficiencies than Trx2 and NrdH, respectively. Interestingly, the catalytic efficiency for the TR1-Trx1 couple is almost five-fold lower than the equivalent from E. coli (196).

When all of the potential redoxins were screened as electron donors for B. anthracis RNR, only Trx1 and NrdH were active with the manganese form of the enzyme, whereas none of them stimulated the activity of the iron form of RNR. This strengthens the notion that type Ib RNRs are indeed manganese containing enzymes (72–74, 76, 79). When Trx1 and NrdH were compared as electron donors for RNR, Trx1 was more efficient, giving a seven-fold higher catalytic efficiency, primarily due to a six-fold lower $K_m$. This is in contrast to the situation in E. coli, where NrdH, but not Trx1 was active as electron donors for its type Ib RNR (119).

To be able to draw relevant conclusions about the physiological roles of the different active redoxins, we needed information about relative levels. First we studied the relative enzymatic activities in lysates of the non-pathogenic strain B. anthracis Sterne 7700 (pOX1’, pOX2’), by incubating the lysates with polyclonal antibodies raised against the respective pure proteins. We established that close to 100% of the enzymatic activity could be attributed to Trx1. To confirm this, we developed quantitative western-blot which showed that Trx1 was present at 15 and 60 times higher concentrations than Trx2 and NrdH, respectively.

Although essentiality of TR1 and Trx1 has not been formally proven in B. anthracis, this seems highly likely given results presented in our investigation and the essentiality of corresponding genes in related species (89, 161, 165). Based on this we suggested that TR1 and Trx1 could constitute attractive drug targets.
4.3 PAPER III

Ebselen (2-phenyl-1,2-benzoiselenazol-3(2H)-one, Figure 6) was developed as a glutathione peroxidase mimic (197) and has been evaluated in phase III clinical trials against acute ischemic stroke by the Daiichi Pharmaceutical Company, Japan. It showed a favorable safety profile although the treatment effect was not statistically significant (198). In 1996, when mammalian TR was discovered to be a selenoenzyme, a collaboration was initiated involving Daiichi and the Holmgren laboratory. Ebselen was shown to be a substrate for mammalian thioredoxin reductase, allowing for TR-dependent detoxification of hydroperoxides. It also acted as a superfast oxidant of thioredoxin (199, 200). Furthermore, results from this laboratory in 1996, showed that ebselen acted as an inhibitor rather than a substrate for E. coli TR. This gave rise to the hypothesis that ebselen could act as an antibacterial agent through inhibition of bacterial TRs.

![Structure of ebselen](image)

*Figure 6. Structure of ebselen (2-phenyl-1,2-benzoiselenazol-3(2H)-one)*

In material provided by Daiichi, ebselen had been found to have antibacterial properties against selected bacteria with MIC values down to 0.2 µg/ml for S. aureus and a rapidly bacteriocidal mode of action through an unknown mechanism (201).

We observed that most of the bacteria sensitive to ebselen lack glutathione. In combination with the finding that ebselen inhibits E. coli TR, a mechanism where ebselen exerts its action through inhibition of bacterial thioredoxin reductases in bacteria devoid of glutathione became likely.

When we tested ebselen as an inhibitor of E. coli TR, we found that it acted as a competitive inhibitor with a $K_i$ of 0.5 µM targeting the C-terminal active site cysteine (C138). The sulfur analog of ebselen, ebsulfur, where the selenium atom has been substituted for a sulfur atom was instead a substrate for the E. coli enzyme whereas it acted as an inhibitor of the thioredoxin reductase from H. pylori. This difference was interesting, not least given the small, but distinct differences found between the two enzymes in paper I (185).
In an attempt to dissect the mechanism, we utilized different *E. coli* strains with various null-mutations in the thioredoxin and glutaredoxin systems. We could see that strains with deletions in the systems for synthesis or regeneration of glutathione were more sensitive to exposure to ebselen than the strains in which parts of the thioredoxin system had been deleted.

To further corroborate our hypothesis, we exposed different logarithmically growing *E. coli* strains to ebselen and could show that growth-arrest correlated with *in vivo* oxidation of thioredoxin 1 in strains unable to produce glutathione.

In the last part of the paper, we expanded the set of sensitive bacteria with *H. pylori* and *M. tuberculosis*, both of which are unable to synthesize glutathione.

The combined data demonstrate that ebselen is a competitive inhibitor of *E. coli* TR *in vitro* acting by binding to the active site cysteines. Furthermore, that inhibition of TR and direct oxidation of Trx might be important mechanisms of action for ebselen *in vivo*, in bacteria devoid of glutathione.
In this paper, we took the next logical step and characterized ebselen and derivatives thereof as inhibitors of \textit{B. anthracis} thioredoxin reductase.

When we compared the sequences of TR from \textit{B. antracis} with some other members of the low-GC group of Gram-positive bacteria, we could see that the degree of conservation was high with 86\% identity for \textit{B. subtilis} and 73\% identity for \textit{S. aureus}. The identity was lower for the Gram-negative bacterium \textit{E. coli} which exhibited 38\% identity.

Ebselen was verified as an inhibitor of \textit{B. anthracis} TR with a IC50 value of 1.0 µM assayed with 25nM TR and 2.5µM Trx1 and DTNB as the terminal substrate. When a series of 12 analogs were evaluated, IC50 values varied from 70nM to 14.8µM. An IC50 value of 70 nM, as observed for the most potent compound (compound nr 12, please refer to manuscript IV for structures), represents a close to stoichiometric concentration with respect to available active sites in TR. The second most potent inhibitor of the enzyme was compound nr 9 which gave an IC50 value of 370nM. This represents close to a three-fold increase in potency relative to parental ebselen.

In the next part of the study, we tested the compounds against \textit{B. subtilis} as a substitute for \textit{B. anthracis}, which requires special considerations for use. The compounds were evaluated based on two fundamental parameters – MIC and MBC. The MIC (Minimal Inhibitory Concentration) is defined as the lowest concentration of drug preventing all visible growth of bacteria. MBC (Minimal Bacteriocidal Concentration) is defined as the lowest concentration of drug resulting in a minimum of 99.9\% reduction in CFU (Colony Forming Units) during 24 hours, where a CFU is assumed to represent one viable bacterium in the culture. The MIC value was compared to the previously established IC50 value for a human cell line (HEK 293) (202) and selectivity indices (SI) were calculated by dividing the IC50 for HEK cells with the MIC for \textit{B. subtilis}.

The MIC values were found to vary between 0.4µM (0.12µg/ml) and >16µM. The most potent group of compounds, with a MIC of 0.4-0.5µM included parental ebselen, compound nr 9 and compound nr 12, but also a 2-methyl, 4-cloro substituted derivative (compound nr 3). MBC values were found at 1-2 times the MIC values, suggesting a general bacteriocidal mode of action. Selectivity indices were found to vary between <3.4 and >400. The group of compounds with the highest selectivity indices (>400 – 160) included compounds 1, 3 and 9. Notably, compound nr 12 gave a modest SI of 25.
On a general note, inhibitory activity on the pure enzyme showed a reasonably good correlation to the effects seen on *B. subtilis*, although exceptions exist. For example, compounds with carboxylic acid substituents gave reasonable IC50 values, which were 4.5 – 5 times higher than parental ebselen, but MIC values which were >32 times higher. Since these carboxylic acid substituents are likely de-protonated and negatively charged under physiological pH, we speculate that this might impair permeability. Furthermore, compound 12, which was 15-fold more potent as an inhibitor of the pure enzyme gave the same MIC value as parental ebselen. This might be attributable to decreased permeability due to the larger size of the molecule.

The most powerful technique to obtain structural information about a protein is X-ray crystallography. Structural information can potentially be used for rational design and/or optimization of inhibitors (31). Therefore, a long-standing goal has been to crystallize a complex between a bacterial TR and ebselen. However, co-crystallization of preformed complexes between TRs from different species and ebselen has hitherto been unsuccessful. The same goes for post-crystallization soaking of crystals (data not shown).

Therefore, we decided to characterize the complex between the C135S mutant of *B. anthracis* TR and ebsulfur where ebsulfur is an analog of ebselen in which the selenium atom has been substituted for with sulfur. The most important reason for choosing ebsulfur over ebselen, is the somewhat higher solubility of the former.

Mass-spectrometry verified close to complete complex formation between ebsulfur and the protein in a 1:1 stoichiometry. The complex eluted as a nice symmetrical peak in size-exclusion chromatography which should be a good starting point for crystallization. A good behavior in size-exclusion chromatography appears to have a positive predictive value for crystallization success (203).

All in all, the most promising compound was compound nr 9, which gave the second lowest IC50 value (370 nM) for the enzyme, the lowest MIC (0.4µg/ml) and the best selectivity index (>400). Hopefully this or another analog based on the structure-activity-relationship established in this study can be developed into an antibiotic for the treatment of anthrax. Given the high degree of similarity between TRs in the particular group of bacteria, data can likely be extrapolated to other bacteria such as *S. aureus* – at least on an enzymatic level.
5 CONCLUDING REMARKS

Since antimicrobial resistance is a major problem and continued development of new antibiotics is needed, a few important questions arise:

- Are bacterial thioredoxin systems good drug targets?
- Does ebselen represent an attractive drug candidate for further development?
- Is the choice of *H. pylori* and *B. anthracis* as primary study objects adequate?

The essentiality criteria is reasonably well fulfilled for representative bacteria (*B. subtilis, S. aureus* and to a lesser extent *H. pylori*). There seems to be a general trend that bacteria devoid of glutathione are heavily dependent on thioredoxin and thioredoxin reductase for synthesis of DNA and protection against oxidative stress.

Thioredoxin reductase is present in the host (humans), but is dispensible (at least in mice) after birth, due to the presence of the glutaredoxin system. Furthermore, thioredoxin reductase is sufficiently different in mammals and bacteria to allow for selective targeting.

With regards to the possible spectrum of activity for an inhibitor, it includes distinct members from different groups such as Gram-negatives (including as *H. pylori*), Gram-positives (including *S. aureus* and *B. anthracis*) and acid fast bacilli (including *M. tuberculosis*). Interestingly, a large part of the normal flora (including *E. coli*) does have glutathione which could allow for selective targeting with fewer side effects.

- Bacterial thioredoxin systems are good drug targets in bacteria devoid of glutathione (II-IV).
- Species/group specific differences with respect to structure and function of TRs and Trxs need to be taken into account when evaluating inhibitors (I, III, IV).

When ebselen is considered as a potential antibiotic lead compound, several things speak in its advantage. The favourable toxicity profile of ebselen as a phase III drug against ischemic stroke is encouraging and its ability to work as an antioxidant in mammalian systems represent a possible added bonus. The favourable selectivity indices are also encouraging as well the novel mechanism of action.

- Ebselen and analogs thereof represent attractive leads for antibiotic development (III, IV).
The need for development of novel antibacterial targeting *H. pylori* is warranted on the direct basis of its common occurrence as a causative agent behind peptic ulcer disease and the increasing resistance seen amongst clinical isolates.

As for *B. anthracis*, the question is a bit harder given that there are only about 5000 cases annually, worldwide. Thus, it can hardly be considered a major public health problem as of today. However, antibiotic development should ideally be one step ahead. Furthermore, the ease with which resistant strains can be generated in the laboratory is problematic. Additionally, for systems which are highly conserved between *B. anthracis* and other bacteria, extrapolation to other species might be possible.

*H. pylori* and *B. anthracis* represent the opposite sides of the spectrum with respect to many basic physiology features (such as Gram-stain, sporulation ability and oxygen tolerance/requirements) and the type of disease they cause (slow, chronic infections vs rapidly progressing).

- Continued development of antibiotics targeting *H. pylori* and *B. anthracis* is warranted.

- *H. pylori* and *B. anthracis* are interesting species from a redox perspective and especially the thioredoxin systems deserve investigation given the absence of glutathione (I–IV).

- Data obtained using the thioredoxin system from *B. anthracis* can likely be extrapolated to other bacteria such as *S. aureus* (II and IV).

All in all, the data presented herein strongly suggest that bacterial thioredoxin systems represent good drug targets in bacteria devoid of glutathione. Furthermore, ebselen represents an interesting starting point for development of a novel class of antibacterials. This class could potentially be used to treat a wide variety of infections including anthrax, peptic ulcers and tuberculosis.
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7 REFERENCES


    M., Koren, S., Okinaka, R. T., Chung, W.-K., Schupp, J. M., Wagner, D. M.,
    Vipond, R., Foster, J. T., Bergman, N. H., Burans, J., Pearson, T., Brooks, T.,
    and Keim, P. (2012) Molecular epidemiologic investigation of an anthrax
    outbreak among heroin users, Europe. *Emerging Infect. Dis.* 18, 1307–1313
    *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus
    mycoides* from a range of clinical and environmental sources as determined by
55. Inglesby, T. V., O'Toole, T., Henderson, D. A., Bartlett, J. G., Ascher, M. S.,
    Eitzen, E., Friedlander, A. M., Gerberding, J., Hauer, J., Hughes, J., McDade,
    J., Osterholm, M. T., Parker, G., Perl, T. M., Russell, P. K., and Tonat, K.
    management. *JAMA* 287, 2236–2252
    overview of anthrax infection including the recently identified form of disease
    in injection drug users. *Intensive Care Med* 38, 1092–1104
57. Athamna, A., Athamna, M., Abu-Rashed, N., Medlej, B., Bast, D. J., and
    Rubinstein, E. (2004) Selection of *Bacillus anthracis* isolates resistant to
59. REICHARD, P., and ESTBORN, B. (1951) Utilization of desoxyribosides in
60. REICHARD, P., BALDESTEN, A., and RUTBERG, L. (1961) Formation of
    deoxycytidine phosphates from cytidine phosphates in extracts from
    *Escherichia coli*. *J. Biol. Chem.* 236, 1150–1157
    Formation of deoxycytidine diphosphate from cytidine diphosphate with
    enzymes from *Escherichia coli*. *J. Biol. Chem.* 237, 3513–3519
    3488
    tyrosine free radical in ribonucleotide reductase from *Escherichia coli*. *J. Biol.
    Chem.* 253, 6863–6865
64. Stubbe, J., and Van der Donk, W. A. (1998) Protein radicals in enzyme
    Biochem* 67, 71–98
    Biochem* 75, 681–706
    63
    curated database of the universal enzyme family ribonucleotide reductase,
    reveals a high level of misannotation in sequences deposited to Genbank. *BMC
    Genomics* 10, 589
75. Martin, J. E., and Imlay, J. A. (2011) The alternative aerobic ribonucleotide reductase of Escherichia coli, NrdEF, is a manganese-dependent enzyme that enables cell replication during periods of iron starvation. Molecular Microbiology 80, 319–334


100. Laurent, T. C., Moore, E. C., and Reichard, P. (1964) Enzymatic synthesis of deoxyribonucleotides. IV. Isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J. Biol. Chem.* **239**, 3436–3444


calf thioredoxin reductase are homologous to glutathione reductase with a
carboxyl-terminal elongation containing a conserved catalytically active
penultimate selenocysteine residue. J. Biol. Chem 273, 8581–8591

mammalian thioredoxin reductase: the active site is a redox-active
selenolthiol/selenenylsulfide formed from the conserved cysteine-


from biology to health and disease. Antioxid. Redox Signal 9, 25–47

Semin. Cancer Biol. 16, 420–426


134. Jakupoglu, C., Przemeck, G. K. H., Schneider, M., Moreno, S. G., Mayr, N.,
Hatzopoulos, A. K., De Angelis, M. H., Wurst, W., Bornkamm, G. W.,
essential for embryogenesis but dispensable for cardiac development. Mol. Cell.

O., Merrill, G. F., Prigge, J. R., Siders, A. M., Wakamiya, M., Wallin, S. L.,

Comstock, K. M., Suvorova, E. S., Capecci, M. R., Merrill, G. F., and
Schmidt, E. E. (2010) Hepatocytes lacking thioredoxin reductase 1 have normal
replicative potential during development and regeneration. J. Cell Sci. 123,
2402–2412

137. Prigge, J. R., Eriksson, S., Iverson, S. V., Meade, T. A., Capecci, M. R.,
growing liver requires either glutathione or a single allele of txnrd1. Free Radic.
Biol. Med. 52, 803–810

Acad. Sci. U.S.A 73, 2275–2279

Purification and characterization of glutaredoxin from Escherichia coli. J. Biol.
Chem. 254, 3664–3671

Characterization of the enzymatic mechanism of Escherichia coli glutaredoxin.
J. Biol. Chem. 254, 3672–3678

141. Xia, B., Vlamis-Gardikas, A., Holmgren, A., Wright, P. E., and Dyson, H. J.
(2001) Solution structure of Escherichia coli glutaredoxin-2 shows similarity to
mammalian glutathione-S-transferases. J. Mol. Biol. 310, 907–918

dependent redox enzymes with functions far beyond a simple thioredoxin
backup system. Antioxid. Redox Signal 6, 63–74


