Universitat Autònoma de Barcelona

Departament de Genètica i de Microbiologia

Institut de Biotecnologia i de Biomedicina

Towards an Understanding of Ribonucleotide Reduction in Bacteria: a comprehensive study in *Streptococcus pyogenes* and *Escherichia coli*

Ignasi Roca Subirà

2007

Universitat Autònoma de Barcelona

Departament de Genètica i de Microbiologia

Institut de Biotecnologia i de Biomedicina

Towards an Understanding of Ribonucleotide Reduction in Bacteria: a comprehensive study in *Streptococcus pyogenes* and *Escherichia coli*

Memòria presentada per el llicenciat en Biologia Ignasi Roca Subirà per optar al grau de Doctor en Ciències Biològiques per la Universitat Autònoma de Barcelona.

Vist-i-plau

Els directors de la tesis

Dr. Isidre Gibert Gonzàlez

Dr. Eduard Torrents Serra

Bellaterra, Juny 2007

Hello darkness, my old friend,
I've come to talk with you again,
Because a vision softly creeping,
Left its seeds while I was sleeping,
And the vision that was planted in my brain,
Still remains,
Within the sound of silence.

In restless dreams Iwalked alone,
Narrow streets of cobblestone,
Neath the halo of a street lamp,
I turned my collar to the cold and damp,
When my eyes were stabbed by the flash of
A neon light
That split the night,
And touched the sound of silence.

And in the naked light I saw,
Ten thousand people maybe more,
People talking without speaking,
People hearing without listening,
People writing songs that voices never share,
And no one deared,
Disturb the sound of silence.

"Fools" said I, "you do not know,
Silence like a cancer grows,
Hear my words that I might teach you,
Take my arms that I might reach you",
But my words like silent raindrops fell,
And echoed,
In the wells of silence.

And the people bowed and prayed,
To the neon God they made,
And the sign flashed out its warning,
In the words that it was forming,
And the signs said, "the words of the prophets
Are written on the subway walls,
And tenement halls".
And whispered in the sounds of silence.

Simon & Garfunkel, The Sound of Silence

Als meus Pares,
Carlos i Nino

Summary

Ribonucleotide reductases (RNRs) are a group of enzymes that catalyze the reduction of ribonucleotides (NTPs) to deoxyribonucleotides (dNTPs), thus providing all organisms with optimal amounts of the necessary buildings blocks for DNA replication and repair. Three classes of RNRs have been characterized up to date according to their different mechanisms for radical generation, structural differences, allosteric regulation and oxygen dependence, all of them having in common the reaction mechanism and the use of an organic free radical to initiate catalysis.

The genome of *Streptococcus pyogenes* (Group A Streptococcus [GAS]), a human pathogen responsible for many diverse infections, harbours the genes to synthesize two complete class Ib RNRs (*nrdHEF/nrdI2* and *nrdFIE** respectively), while most streptococci only bear the *nrdHEF/nrdI2* genes. This work attempts to determine whether the presence of a second class Ib RNR in *S. pyogenes* accounts for a biological need or if it is simply the result of a vestigial burden. *S. pyogenes* is the first prokaryotic organism where two distinct and complete ribonucleotide reductases of the same class are studied.

Linkage RT-PCR showed that both clusters are simultaneously transcribed and constitute two independent polycistronic units. The α and β subunits for these two systems were purified and biochemically characterized. The first system (*nrdHEF*) proved active and presented an EPR signal that matched that of other class Ib tyrosyl radicals. Activity was dependent on the addition of magnesium and DTT and required NDPs but not NTPs as a substrate. It was also stimulated by dATP as an allosteric effector but not by ATP, as expected in a class Ib enzyme. However, neither activity nor tyrosyl radical signal were ever detected from the *nrdFIE** cluster. When aligned together with other class Ib enzymes, three out of the six iron binding residues within the β subunit of the *nrdFIE** cluster were found not to be conserved. The iron contents of the two NrdF proteins clearly showed that, contrary to the β subunit from *nrdHEF*, the β subunit from *nrdFIE** was not capable of coordinating an iron centre *in vitro* under the conditions tested, which, in turn, impaired the generation of a tyrosyl radical.

Heterologous complementation assays, however, showed that the *nrdFIE** operon is fully functional *in vivo* when all three genes are present. In a similar manner, the *nrdHEF* system showed no complementation unless a functional NrdI protein was provided. Neither NrdI nor NrdI2 from *S. pyogenes* rendered *nrdHEF* active, but it did addition of the NrdI2 protein form *S. pneumoniae*. The presence of an almost identical *nrdFIE* cluster in all *Mycoplasma* species leads us to suggest that the *nrdFIE** operon was acquired by *S. pyogenes* through a lateral gene transfer event to compensate for the loss of a functional NrdI.

We have also studied the promoter regulating the transcriptional expression of the anaerobic ribonucleotide reductase of *Escherichia coli* (*nrdDG*). This promoter has been shown to be up-regulated by the pleiotropic transcriptional regulator FNR (fumarate and nitrate reduction), and two regions with significant similarity to the FNR consensus sequence were also found. In this work we have investigated the binding of FNR to the *nrdDG* promoter region and the effects of such binding on transcription. Gel retardation analysis with purified FNR* demonstrated FNR interaction at both sites, and studies with altered FNR boxes indicated that the upstream FNR-2 site is essential for the anaerobic activation of the *nrdDG* promoter. The FNR-1 site, however, is needed for the maximal expression of this promoter. Our results suggest that both sites act synergistically to coordinate expression in response to shifting oxygen concentrations.

Index

Abbreviations

(d)ADP	(deoxy) Adenosine diphosphate
(d)ATP	(deoxy) Adenosine triphosphate
(d)CDP	(deoxy) Cytidine diphosphate
(d)CTP	(deoxy) Cytidine triphosphate
(d)GDP	(deoxy) Guanosine diphosphate
(d)GTP	(deoxy) Guanosine triphosphate
(d)NDP	5'-(Deoxy)ribonucleoside diphosphate
(d)NTP	5'-(Deoxy)ribonucleoside triphosphate
(d)TDP	(deoxy) Thymidine diphosphate
(d)TTP	(deoxy) Thymidine triphosphate
2' or 3'	2' or 3' carbon
A	Adenine or adenosine
Å	Angstrom units
aa	aminoacid
AdoCbl	5'-deoxyadenosylcobalamine
AdoMet	5'-deoxyadenosylmethionine
Apo R2	R2 apoprotein. It contains neither iron nor radical
Arc	Aerobic respiration control
bp	Base pair
C	Cytosine or cytidine
CFU	Colony forming units
cpm	Counts per minute
C-term	Carboxyterminal end
Da	Daltons
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EPR	Electron paramagnetic resonance
$FAD^{+}(H_2)$	Flavin adenine dinucleotide (reduced form)

FIS	Factor for inversion stimulation
FMN	Flavin mononucleotide
FNR	Fumarate nitrate reductase
FPLC	Fast protein liquid chromatography
FUR	Ferric uptake regulator
G	Guanine or guanosine
Grx	Glutaredoxin
GSH and GSSG	Reduced glutathione and its corresponding oxidized form
HU	Hydroxyurea
IPTG	Isopropyl-ß-D-thiogalactopyranoside
Kbp	Kilo base pair
KDa	Kilo dalton
Km	Michaelis-Menten constant
Met R2	R2 protein containing two ferric irons but no radical
mRNA	Messenger RNA
$NADPH^{+}(H)$	Nicotinamide adenine dinucleotide phosphate (reduced form)
nrd	Nucleotide reduction genes
NrdAB	Class Ia RNR large and small subunits
NrdDG	Class III RNR large and small subunits
NrdEF	Class Ib RNR large and small subunits
NrdJ	Class II RNR
N-term	Aminoterminal end
PCR	Polymerase chain reaction
PFL	Piruvate formate lyase
PMSF	Phenylmethylsulfonyl Fluoride
R1	Class I RNR large subunit
R2	Class I RNR small subunit
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNR	Ribonucleotidil reductase
RT-PCR	Reverse transcriptase-PCR
S·	Thiyl radical

SAM	S-adenosylmethionine
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	Thyol group
T	Thymine or thymidine
T·	Tyrosyl radical
Trx	Thioredoxin
ts	Temperature-sensitive
U	Uracyl or Uridine
UDP	Uridine diphosphate

Aminoacids

Ala (A)	Alanine
Arg (R)	Arginine
Asn (N)	Asparagine
Asp (D)	Aspartic acid
Cys (C)	Cysteine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Gly (G)	Glycine
His (H)	Histidine
Ile (I)	Isoleucine
Leu (L)	Leucine
Lys (K)	Lysine
Met (M)	Methionine
Phe (F)	Phenylalanine
Pro (P)	Proline
Ser (S)	Serine
Thr (T)	Threonine
Trp (W)	Tryptophan
Tyr (Y)	Tyrosine
Val (V)	Valine

1. - General Introduction

1.1 - Ribonucleotide Reductases: from RNA to DNA

Life on earth, as we know it today, depends on the properties of self-replication and catalysis of three main interplaying macromolecules: proteins, DNA, and RNA. With the exception of some RNA-based viruses, DNA contains the genetic information that determines the essence of every living organism, which is passed on from one generation to another. In order to generate a living cell, the stored information must be translated and used to synthesize either catalytic or structural proteins, being RNA (mRNA) the molecule that provides the link between the stored information (DNA) and the biosynthetic machinery of the cell. Such a flow of genetic information defines what nowadays is known as the ”DNA world”.

It is thought, however, that early during the evolution of life, there was a time where DNA and proteins were not yet present and RNA constituted the only molecule to provide the means for both self-replication and catalysis. Proteins developed at a later stage and proved a better catalyst than RNA, which became the main storage of genetic information as well as the linking molecule to the protein machinery. The recognition that RNA had properties that are so central to the creation of life gave rise to the “RNA world” hypothesis (Gilbert, 1986) (Fig. 1).

As the main repository of hereditary information, RNA is a quite unstable molecule and has a poor fidelity of replication. During the early stages of life, these features might have been an advantage since a higher mutation rate provided a greater amount of variants that allowed a more rapid evolution. On the long run, however, such a low fidelity of replication prevents an accurate transmission of the genetic information and constrains the long-term stability of the fittest variants.

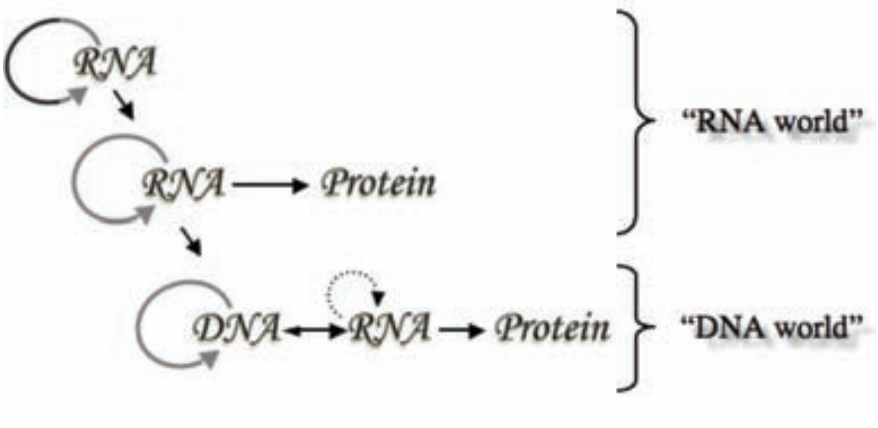

Figure 1. Schematic model representic the flow of genetic information across the evolution of life

There are two main characteristics that contribute to the instability and low fidelity of RNA:

First, RNA contains a hydroxyl group at the 2' position of the ribose ring. At a low but significant frequency, the 2' oxygen of this group successfully attacks the phosphate bonded to the 3' position, "stealing" the bond from the neighbouring base and forming a cyclic 2'-3' phosphodiester bond that severs the chain of nucleotides. This reaction is enhanced by high pH, and is also encouraged by many enzymes that degrade RNA (Larralde *et al.*, 1995).

Second, RNA uses uracil as the partner of adenine instead of thymine. The difference between the two pyrimidines is chemically fragile; at the 4' position, cytosine has an amino group while uracil has a double bonded oxygen and, at an appreciable frequency, water molecules can interact with the carbon housing the amino group, de-aminating it and leaving behind a double bounded oxygen. That is, cytosine can be easily converted into uracil, with drastic consequences for the information contained within the RNA molecule (Nelson & Cox, 2000).

At this point, it is clear that a new and more stable repository was needed to ensure the fidelity of the hereditary information and, since most of the inconveniences of RNA were related to the presence of the 2'-hydroxyl group, the new repository might easily evolve from RNA by “simply” removing it. This seemingly simple step was not possible until a unique and exceptional enzyme came into play, an enzyme that catalyzed the replacement of the 2'-OH with hydrogen, thus, producing deoxyribonucleotides (dNTPs) from the reduction of

ribonucleotides (NTPs) and, hence, this enzyme was termed Ribonucleotide Reductase (RNR) (Fig. 2).

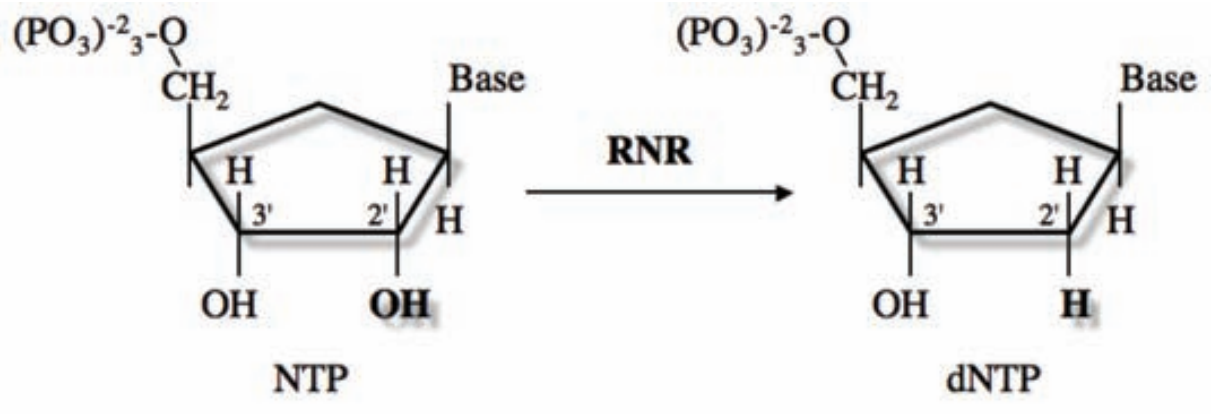

Figure 2. Ribonucleotide reductase specific reduction of the 2´OH group of NTPs to generate dNTPs

The members of the ribonucleotide reductase family of metalloenzymes (EC 1.17.4.1) carry out the only known metabolic pathway for providing any living cell with a pool of dNTPs in what is known as the *de novo* pathway. These enzymes utilize an organic free radical to generate a highly conserved transient active-site thiyl radical to abstract the 2'-OH group from the ribose ring, and they have become the first and best-characterized example of the involvement of free radical chemistry in biochemical reactions.

Furthermore, DNA synthesis and repair not only requires a constant pool of four chemically different deoxyribonucleotides (namely, dATP, dGTP, dCTP, and dTTP), but a balanced supply of each of them as well. In order to respond to such a need, RNRs evolved a novel and highly complex allosteric regulation that governs both the substrate specificity and the overall activity of these enzymes (Nordlund & Reichard, 2006).

Ribonucleotide reductases provided the building blocks for a much more stable molecule, DNA, by eliminating an unwanted side reaction. They became the key enzymes that allowed the transition from the RNA to the DNA world.

Back in the 1960s, Peter Reichard and co-workers discovered and characterized the first RNR in the enteric bacterium *Escherichia coli* (Reichard & Rutberg, 1960), and half a century later ribonucleotide reduction has become a challenging field involving several

research groups which have broaden our understanding of these remarkable proteins, unravelling their biochemical and genetic diversity, and formulated new and breathtaking dilemmas.

1.2 - Classes of RNRs

Besides their essential role in every living cell, RNRs are also of great interest due to their unique biochemical properties, which are used to classify them in three major classes according to their different subunit composition, allosteric regulation, dioxygen sensitivity, cofactor requirements and radical generating mechanisms (Table 1), yet all of them with a common reaction mechanism and the use of an organic free radical to initiate the reduction, in just one active site, of both purine and pyrimidine ribonucleotides (Nordlund & Reichard, 2006).

Class I enzymes are found in all eukaryotes, animal viruses as well as in some prokaryotes and bacteriophages (Torrents *et al.*, 2002) and they use an iron-containing protein for the generation of the catalytically essential free radical. In these reductases, the quaternary organization of the holoenzyme is $\alpha_2\beta_2$ (this is the currently accepted organization, but the overall structure of class I enzymes is still an open debate). The α_2 dimer, called R1, contains both the active sites and the allosteric regulatory sites, while the β_2 dimer, called R2, contains one dinuclear iron center and one putative stable tyrosyl radical per monomer, both essential for enzymatic activity (Eklund *et al.*, 2001). Most of the biochemical studies on class I RNRs have been performed on the *Escherichia coli* (Fontecave *et al.*, 1992; Nordlund & Eklund, 1993) and mouse enzymes (Thelander & Graslund, 1993), which are, therefore, regarded as models for class I RNRs (Table I).

The organic free radical of R2 have been shown to be located at Tyr122 (*E. coli* numbering) (Larsson & Sjoberg, 1986) and it is generated by molecular oxygen in close cooperation with the dinuclear iron center (Sahlin *et al.*, 1987). Reduction of the substrate is performed by a pair of cysteines located at the active site of R1, which becomes oxidized during the reaction. In order to reduce back these oxidized cysteines and, therefore, enable another cycle of substrate reduction, class I enzymes utilize thioredoxin and glutaredoxin based systems which are in turn reduced by thioredoxin reductase and glutathione, respectively (Holmgren, 1989). The ultimate reductant in both systems is NADPH. For class I enzymes, oxygen is a component of the system that generates the tyrosyl radical and, therefore, these enzymes are oxygen-dependent.

Table I. Distinguishing features of ribonucleotide reductases (Adapted from Jordan & Reichard, 1998)

	Class Ia	Class Ib	Class II	Class III
Oxygen dependence	Aerobic	Aerobic	Aerobic/Anaerobic	Anaerobic
Structure	$\alpha_2\beta_2/\alpha_?\beta_?$	$\alpha_2\beta_2$	α or α_2	$\alpha_2(\beta_2)$[a]
Gene	*nrdAB*	*nrdEF*	*nrdJ*	*nrdDG*
Radical	Tyr….Cys	Tyr….Cys	AdoCbl….Cys	AdoMet..Gly..Cys
Metal site / cofactor	Fe-O-Fe	Fe-O-Fe	AdoCbl	Fe-S Adomet
Substrate	NDP	NDP	NDP or NTP	NTP
Reduction system	Thioredoxin Glutaredoxin	NrdH-redoxin Glutaredoxin	Thioredoxin	Formate
Allosteric-sites / polypeptide	2	1	1 or 2	2 (1)
dATP inhibition	Yes	No	No	Yes
Distribution	Eukaryote Bacteria Bacteriophage Viruses	Bacteria Bacteriophage	Archaebacteria Bacteria Bacteriophage Some lower-eukaryotes	Archaebacteria Bacteria Bacteriophage
Prototype	*E. coli* Mouse	*S. typhimurium*	*L. leichmannii* *T. maritima*	*E. coli* T4

[a]. Strictly speaking, the active form is an α_2 dimer. The β subunit is the activase used to generate the glycyl radical.

Class I RNR is further subdivided into class Ia and class Ib according to differences in sequence, regulation and reduction (Jordan *et al.*, 1996b; Jordan *et al.*, 1997).

Class Ia is present in eukaryotes and in some bacteria, while class Ib has been only found among bacteria. A distinguishing feature of all known class Ib enzymes is that their α subunit lacks approximately 50 amino-terminal residues which in class Ia are needed to constitute the allosteric activity site (Eliasson *et al.*, 1996).

Class Ib appears to be the active enzyme in those organisms where it is the only aerobic RNR present, as in *Lactococcus lactis* (Jordan *et al.*, 1996b), *Mycobacterium*

tuberculosis (Yang *et al.*, 1994), *Bacillus subtilis* (Scotti *et al.*, 1996), and *Corynebacterium ammoniagenes* (Fieschi *et al.*, 1998).

In those organisms where both classes are present (as in the *Enterobacteriaceae* (Jordan *et al.*, 1994b)), class Ib genes are only weakly transcribed, and mutants defective in the expression of class Ia RNR are not viable despite an increased expression of the second RNR compared to the wild-type organism. Complementation of the lethal mutation, however, can be achieved by addition of one extra copy of the class Ib genes. In contrast, a mutant defective in class Ib expression has no effect whatsoever during aerobic or anaerobic growth (Jordan *et al.*, 1996a) and the reason for those organisms to have two functional class I RNRs still remains unknown. The *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) enzyme constitutes the model enzyme for this class, since it was the first to be reported.

Class II RNRs occur in many prokaryotes as well as in some lower eukaryotes (Torrents *et al.*, 2002), and they are found in bacteria that can live under both aerobic and anaerobic conditions. The enzymes from this class contain just one type of subunit which harbours the active and the allosteric sites, being the monomeric form (α) from *Lactobacillus leichmannii* the best-studied example so far (Licht *et al.*, 1996; Panagou *et al.*, 1972), but as more and more members of this class are being found that are predominantly homodimers (α_2), the *Lactobacillus* enzyme seems to be rather an exception. They all have in common, however, the use of the cobaltous cofactor adenosylcobalamin, a derivative of vitamin B_{12}, to generate a 5'-deoxyadenosyl radical (Ado) (Gerfen *et al.*, 1996) which interacts directly with an active site cysteine to form the reactive cysteine radical needed for ribonucleotide reduction (Booker *et al.*, 1994). As already described for class I, class II enzymes use a thioredoxin-based system as the source of reducing power.

Class II enzymes neither require oxygen nor are inactivated by it, thus, they are oxygen-independent, in contrast to class I and class III RNRs.

Class III enzymes are found in strict, or facultative, anaerobic bacteria and archaea, as well as in some bacteriophages (Torrents *et al.*, 2002). The anaerobic class III reductases use a glycyl radical (Gly580, bacteriophage T4; Gly681, *E. coli*) generated with the aid of S-adenosylmethionine (SAM) together with an iron-sulfur cluster (Eliasson *et al.*, 1990).

The active protein is an α_2 dimer (Torrents *et al.*, 2001) housing the active site, the glycyl radical and both the allosteric sites. The smaller β_2 subunit carries an iron-sulfur cluster

that reacts with SAM to generate the glycyl radical in the large subunit (Sun *et al.*, 1995). The β subunit is also known as the "activase", since it can introduce glycyl radicals into several α subunits (Torrents *et al.*, 2001). The overall reductant is formate (Mulliez *et al.*, 1995) and not an enzyme based system such as in class I and II. Exposure of the active complex to oxygen results in inactivation of the enzyme by cleavage of the polypeptide chain near the glycyl radical, which results in the loss of 25 C-terminal residues (King & Reichard, 1995) and, therefore, class III enzymes are oxygen-sensitive and only active under strict anaerobic conditions.

The anaerobic RNRs from *E. coli* (Barlow *et al.*, 1983) and bacteriophage T4 (Young *et al.*, 1994) are regarded as the model enzymes for this class.

1.3 - Hydrogen Donor Systems

Each ribonucleotide reduction cycle leaves behind a RNR enzyme whose active site contains an oxidized pair of cysteines that needs to be reduced prior to the next turnover cycle. Reduction of this pair of cysteines is achieved by means of external electron donors, which are either small proteins containing an active site with a redox-active disulfide (thioredoxins and glutaredoxins, included in the thioredoxin superfamily) for class I and class II enzymes (Holmgren & Aslund, 1995; Holmgren & Bjornstedt, 1995), or formate for class III enzymes (Mulliez *et al.*, 1995). Thioredoxins (Trx) and glutaredoxins (Grx) catalyse the reversible oxidation of two vicinal protein –SH groups to a disulfide bridge and are present in every living organism, from the simplest prokaryote to humans, taking part in many metabolic processes within the cell (Aslund & Beckwith, 1999; Gleason & Holmgren, 1988). Members of the thioredoxin superfamily (Aslund & Beckwith, 1999; Ritz & Beckwith, 2001) have two features in common:

(i) A short sequence motif that includes a Cys-X_1-X_2-Cys sequence (the active site), which in the oxidized form constitute a disulfide bridge. The active site of thioredoxins is always Cys-Gly-Pro-Cys, while glutaredoxins usually display a Cys-Pro-Tyr-Cys pattern.

(ii) An overall structure containing this motif that corresponds to what is known as a thioredoxin-like fold (Martin, 1995).

Despite sharing these structural properties, the thioredoxin and glutaredoxin families do not share sequence similarities and the sources of electrons required for maintaining their reductive activities differ. The pathway for the reduction of oxidized glutaredoxins initially uses NADPH, which maintains the enzyme glutathione oxidoreductase in the reduced state. This enzyme, in turn, transfers electrons to oxidized glutathione, generating reduced glutathione, which then transfers electrons to oxidized glutaredoxins. In the case of thioredoxins, the initial source of electrons is also NADPH, but in this case the electrons are transferred to the enzyme thioredoxin reductase (TrxB), which then directly reduces oxidized thioredoxins. Finally, each member of the family exhibits its own characteristic redox potential (Aslund *et al.*, 1997).

The best-studied members of the thioredoxin superfamily in *E. coli* are thioredoxins 1 and 2 (*trxA* and *trxC*, respectively) (Miranda-Vizuete *et al.*, 1997; Stewart *et al.*, 1998) and glutaredoxins 1, 2, and 3 (*grxA*, *grxB*, and *grxC*, respectively) (Aslund *et al.*, 1994). It has been shown that thioredoxins 1 and 2 as well as glutaredoxin 1 are capable of reducing the *E. coli* class Ia RNR to regenerate its activity both *in vivo* and *in vitro* (Aslund *et al.*, 1994; Prinz *et al.*, 1997), and only the simultaneous elimination of the three of them produces a lethal phenotype (Stewart *et al.*, 1998). Glutaredoxin 3 exhibits only a low capacity to reduce oxidized class Ia RNR *in vitro*, and it is not able to support the growth of *E. coli* on its own (Aslund *et al.*, 1994) unless a five to eightfold increase in the transcriptional levels of *nrdAB* is achieved (Ortenberg *et al.*, 2004). The electron pathway from NADPH to ribonucleotide reduction is depicted in figure 3.

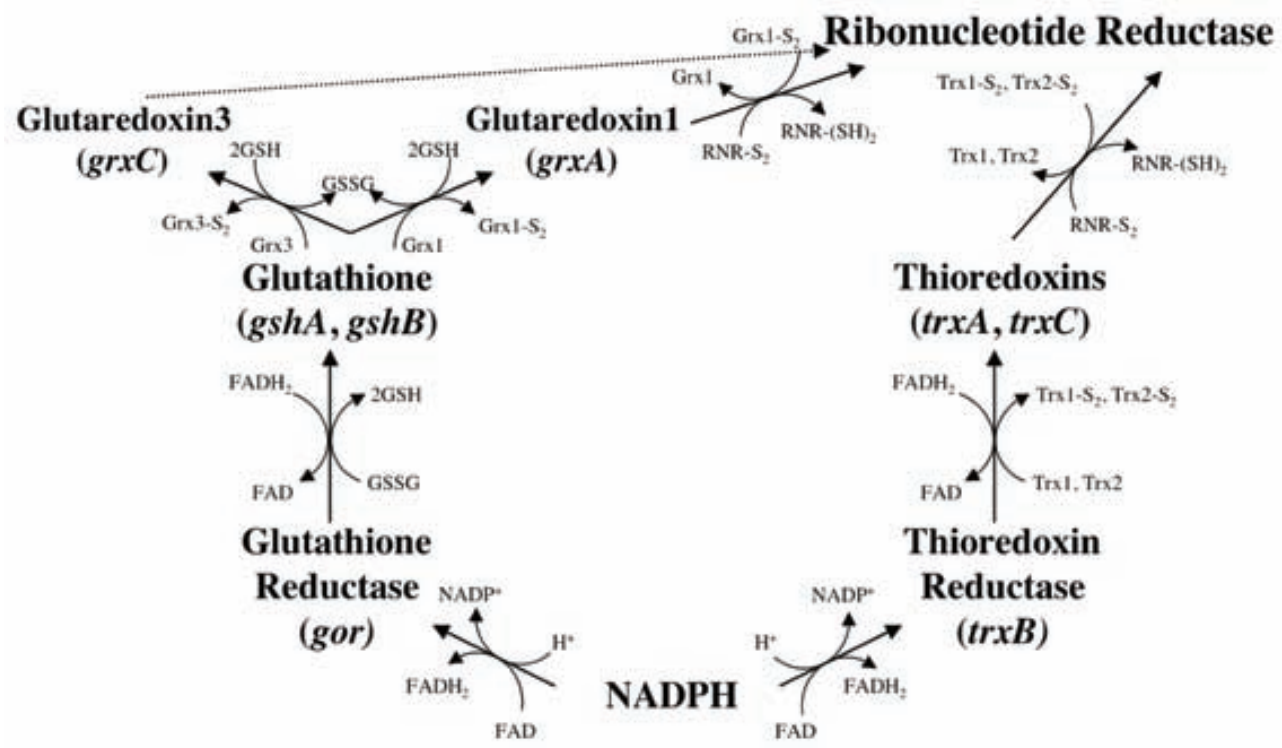

Figure 3. Model representing the physiological hydrogen donor systems in interaction with Class Ia and II RNRs. There are no biochemical evidences showing the channeling of electrons between RNR and Grx3 (dashed arrows). (Adapted from Ortenberg *et al.*, 2004)

In class I RNRs, reduction of one ribonucleotide (ADP, GDP, CDP or UDP) requires two hydrogens that are provided by two cysteines buried within the active site in the *E. coli* R1 (Cys225 and Cys462). The active site cleft, however, is not wide enough to allow a thioredoxin or glutaredoxin to enter and directly reduce the disulfide bridge formed between these two cysteines after substrate reduction (Uhlin & Eklund, 1994). Therefore, there are two additional cysteines (Cys754 and Cys759) at the carboxyl end of R1 that serve as redox shuttles between the active site thiols and the external reductants, normally passing reducing equivalents from thioredoxins or glutaredoxins to the active site cysteines (Aberg *et al.*, 1989). Reduction of the active site cysteines can be accomplished *in vitro* by small dithiolic molecules such as dithiotreitol (DTT). Monothiolic molecules such as 2-mercaptoethanol or glutathione do not show this activity (Reichard, 1962).

Class Ib RNRs use neither thioredoxins nor glutaredoxins as external reductants, instead they use a glutaredoxin-like protein, called NrdH-redoxins (NrdH), which has been found in *E. coli* and several other organisms (Jordan *et al.*, 1996b; Jordan *et al.*, 1997). NrdH proteins are related in amino acid sequence to glutaredoxins but they behave functionally as a thioredoxin. They are not reduced by glutathione but by thioredoxin reductase, they have a low redox potential and they can reduce insulin disulfides; features, all of them, characteristic of thioredoxins (Jordan *et al.*, 1997). Typically, NrdH redoxins are part of a class Ib *nrdHIEF* operon (Gon *et al.*, 2006; Torrents *et al.*, 2002; Torrents *et al.*, 2003).

Class II RNRs only use thioredoxins as external donors, and they share with class I RNRs both the pair of cysteines at the active site and the pair of C-terminal cysteines that shuttle electrons from thioredoxins.

Class III RNR utilize a completely different system to regenerate the active site thiols, instead of using Trx or Grx they use formate as external donor (Mulliez *et al.*, 1995). It is thought that formate binds directly in the active site, where it would pass reducing equivalents to the active site cysteines.

1.4 - Allosteric Regulation

One notable feature of RNRs is their allosteric regulation, which allows a single enzyme to catalyze a balanced reduction of all four ribonucleotides. The specificity for each substrate is determined by the binding of an allosteric effector at a location approximately 15 Å distant from the catalytic site, the so called specificity site or S-site (Reichard, 2002). This regulation of the substrate specificity ensures the production of equal amounts of each dNTP for DNA synthesis and repair, since an unbalanced pool of dNTPs might lead to genetic damage and cell death (Kunz *et al.*, 1994).

The allosteric effectors that bind the S-site are the fully phosphorylated end products of ribonucleotide reduction; dATP (or ATP), dTTP, and dGTP, while dCTP seems to have no effect (Eliasson *et al.*, 1996). Class Ia RNR can be used as a model to illustrate the complex regulation of substrate specificity (Fig. 4): binding of ATP (or dATP) at the S-site stimulates the binding and reduction of CDP and UDP; the end product of such reduction, dTTP, inhibits reduction of CDP and UDP and promotes reduction of the next ribonucleotide, GDP. GDP

reduction produces dGTP, whose binding at the S-site inhibits reduction of GDP and leads to ADP reduction. In general terms, the above mechanism can be applied to the three RNR classes (Jordan & Reichard, 1998).

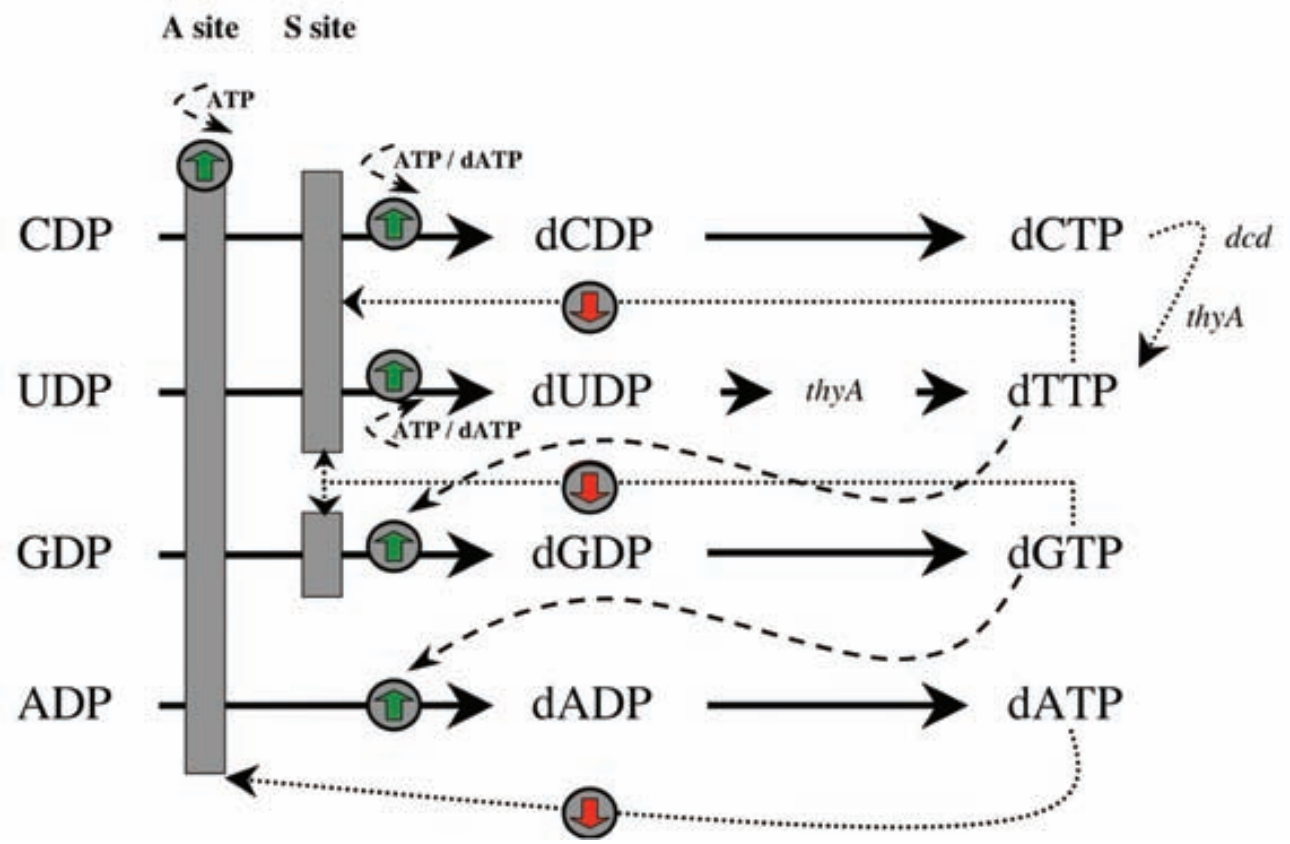

Figure 4. Allosteric Regulation of RNRs. Green upwards arrows indicate stimulation of ribonucleotide reduction, and red downwards arrows indicate inhibition

In most class I and II enzymes, both ATP and dATP promote CDP and UDP reduction since their S-site do not discriminate between one or the other. Furthermore, class Ia and many class III enzymes also possess a second regulatory site, the so called activity site or A-site, which regulates the overall activity of the enzyme (Reichard, 2002). ATP binding at the A-site activates ribonucleotide reduction while binding of dATP shuts it down. Therefore, dATP has both a stimulatory effect and an inhibitory effect; however, since the S-site has a higher affinity for dATP, inhibition is only achieved at high concentrations.

One of the major structural differences between the RNR classes is the lack of approximately 50 amino terminal residues in class Ib and some class II RNRs which, in class Ia, account for the overall activity site (Eliasson *et al.*, 1996). The lack of this amino terminal residues translates in the loss of dATP inhibition. The anaerobic class III enzyme from *E. coli* also contains two types of allosteric sites located in the large α subunit: the purine site, which corresponds to the specificity site in class I and II RNRs, and the pyrimidine site, which corresponds to the activity site in class Ia RNRs (Eliasson *et al.*, 1994b). The anaerobic class III RNR from bacteriophage T4, however, lacks almost 100 amino terminal residues that are present in the *E. coli* class III enzyme, so it does not contain the pyrimidine site (Logan *et al.*, 1999) (Fig. 5).

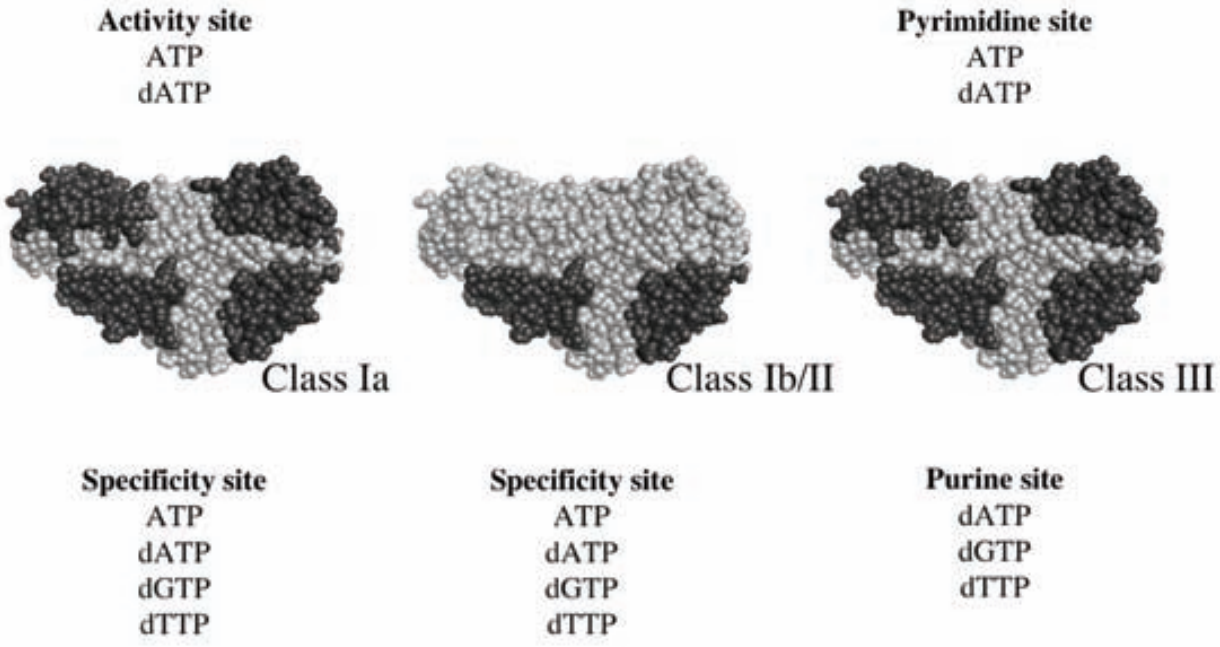

Figure 5. General schematic model of the allosteric sites present in all three RNR classes. (Adapted from Jordan & Reichard, 1998)

Recent studies have proposed a third regulatory site for the mammalian class Ia RNR called the hexamerization site or H-site. The H-site would stimulate the formation of an $R1_6R2_6$ hexamer upon ATP binding, being this hexamer the major active form of RNR in mammalian cells (Cooperman & Kashlan, 2003; Kashlan & Cooperman, 2003). The presence of the H-site introduces a novel regulatory mechanism that controls the oligomerization state

of the enzyme depending on a major or a minor need for deoxyribonucleotides, and can be used to explain the role of the nuclear p53R2 subunit found in mammalian cells (Tanaka *et al.*, 2000). The existence of this novel hexamerization site, however, has many detractors and is yet to be proven.

The specificity sites in class Ia (Eriksson *et al.*, 1997), class Ib (Uppsten *et al.*, 2003) and class III (Larsson *et al.*, 2001) RNRs are found at a four-helix bundle at the dimer interface, suggesting a similar function in these three classes of RNRs. As for the monomeric class II RNR of *L. leichmannii*, the specificity site is proposed to be at a four-helix bundle connected to an additional domain that resembles the dimer interface of other RNRs (Ludwig & Matthews, 2002; Sintchak *et al.*, 2002). This explains how a monomeric enzyme can control substrate specificity as efficiently as a multimeric enzyme, in fact, the majority of currently characterized class II RNRs are dimeric and have effector sites highly homologous to those found in class I (Eliasson *et al.*, 1999) (Fig 6).

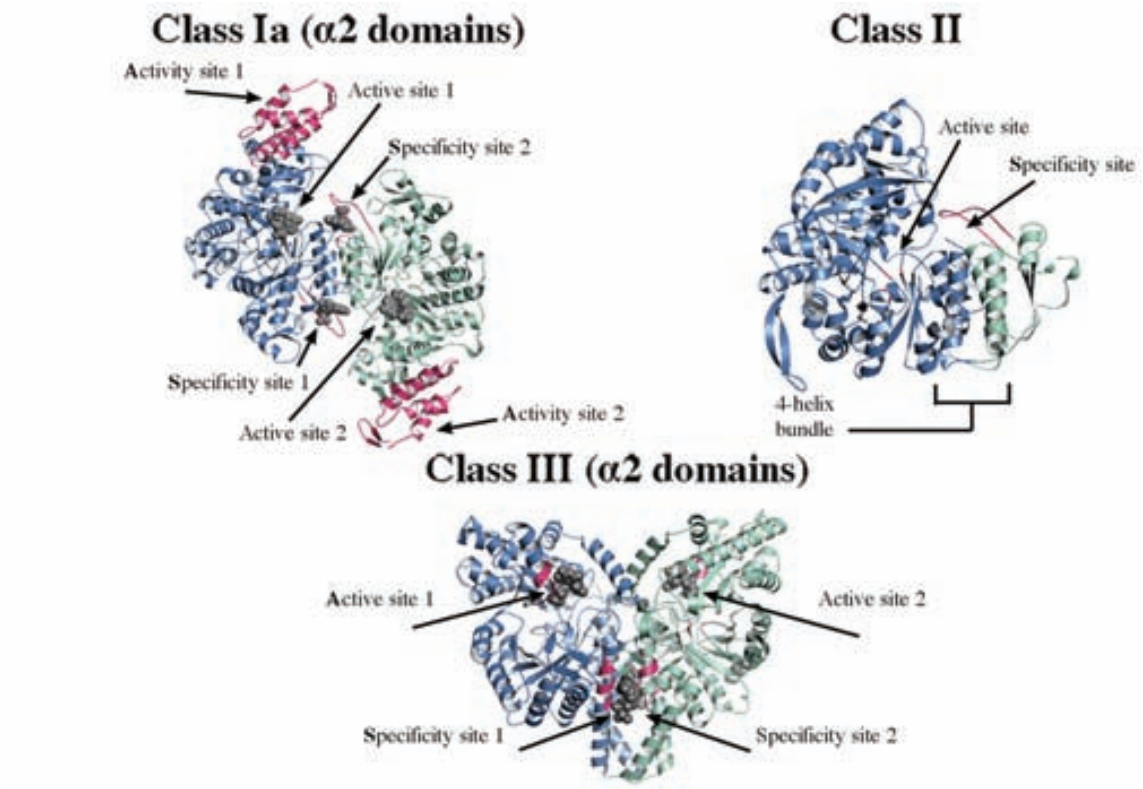

Figure 6. Comparison of allosteric specificity sites in dimeric *versus* monomeric RNRs. Ribbon drawings of Class Ia RNR from *E. coli* (PDB: 4R1R), Class II RNR from *L. leichmannii* (PDB: 1L1L) and Class III from bacteriophage T4 (PDB: 1H79). Dimeric Class Ia and Class III are shown with one chain in blue and one chain

in light green. Monomeric Class II is painted in blue except for the insert domain, which is shown in light green. Specificity, activity and active sites are labelled and shown in magenta for all three classes. (Adapted from Sintchak *et al.*, 2002)

These structural similarities indicate a common pathway to control substrate specificity from a distant allosteric site, but the mechanism itself has not been elucidated yet. Comparison of the structures containing allosteric effectors reveals conformational changes that propagate from the allosteric site to the active site, but it is not clear if this communication is intermolecular (between different α subunits in a dimer) or intramolecular (within a single α subunit). The approximate intramolecular distance between the specificity site and the active site is found to be: 25 Å in class I, 15 Å in class II and 25 Å in class III; and the intermolecular distance for class I RNR is approximately 15 Å. As for the *L. leichmannii* class II RNR, it is obvious that an intramolecular communication is the only option, since it is a monomeric enzyme, but its homology to the head-to-tail dimer interface of class I RNR (Uhlin & Eklund, 1994) suggests an intermolecular information transfer between the α subunits in the R1 dimer (Sintchak *et al.*, 2002). It is worth noticing, however, that binding of R2 might cause conformational changes influencing the nature of this transfer.

Class III RNR, on the other hand, differ from class I and class II RNRs in terms of the topology of the effector-binding region (head-to-head dimer) (Logan *et al.*, 1999), the intermolecular distance between the specificity and active sites and the residues involved in effector binding (Larsson *et al.*, 2001), suggesting an slightly different regulation of substrate specificity.

1.5 - Radical Generation and Metallosites

One of the most fascinating features of RNRs is the use of radical chemistry to initiate catalysis, either by storing a stable protein-derived free radical at some distance from the active site, which is coordinated with a metallosite (a diferric iron cluster in class I (Nordlund *et al.*, 1990; Nordlund & Eklund, 1993; Petersson *et al.*, 1980), and an iron sulfur cluster in class III (Ollagnier *et al.*, 1996)) or by cleaving a B_{12}-cofactor to generate a 5'-deoxyadenosyl radical (Ado) in class II (Gerfen *et al.*, 1996).

1.5.1 - Class I RNR

1.5.1.1 - Class Ia

As mentioned above, class I RNRs utilize a stable tyrosyl radical for the initiation of the catalytic reaction. The structure and generation of this radical have been thoroughly studied in the *E. coli* class Ia enzyme (Larsson & Sjoberg, 1986; Nordlund *et al.*, 1990; Nordlund & Eklund, 1993; Petersson *et al.*, 1980) and, hence, the following discussion will refer to it. The *E. coli* organic radical is generated on a tyrosine residue (Tyr122) close to a diferric iron center with a μ-oxo bridge, and it is deeply buried inside the R2 protein, protected from the solvent by a hydrophobic pocket constituted by conserved residues (namely, Phe208, Phe212 and Ile234) (Nordlund *et al.*, 1990). These conserved hydrophobic residues form a narrow pocket suitable for the access and binding of a dioxygen species to the iron center and provide a hydrophobic and oxidation-resistant environment that protects the radical from the surrounding solvent and ensures that the side chain of Tyr122 is not in van der Waal's contact with any oxidizable side chain.

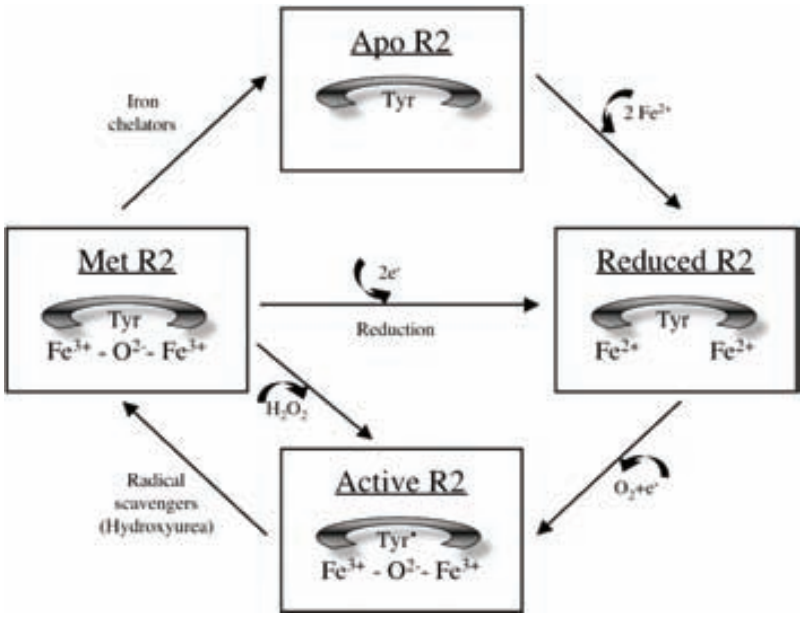

Figure 7. Redox states of the R2 protein from *E. coli*. (Adapted from Sahlin *et al.*, 1989)

The R2 protein can be found in, at least, four different states, depending on the redox states of the two irons and the tyrosine: (i) Active R2 – the active R2 protein, it contains two ferric ions with an μ-oxo bridge and a radical Tyr122; (ii) Met R2 – it is generated when the radical in active R2 is scavenged by single electron reductants, it contains two ferric ions and a normal Tyr122; (iii) Reduced R2 – further reduction of Met R2 yields reduced R2, it contains two ferrous ions and a normal Tyr122; and (iv) Apo R2 – it contains no iron at all and a normal Tyr122 (Fig. 7). The two irons are denoted Fe1 and Fe2 based on their vicinity to Tyr122 and they are coordinated in an octahedral fashion by two histidines (His118, His241), three glutamic acids (Glu115, Glu204, Glu238), one aspartic acid (Asp84), an oxide ion and water molecules. The coordination of Fe2 is close to a perfect octahedron, whereas the coordination of Fe1 is more distorted due to the bidentate binding of Asp84 (Nordlund *et al.*, 1990) (Fig. 8).

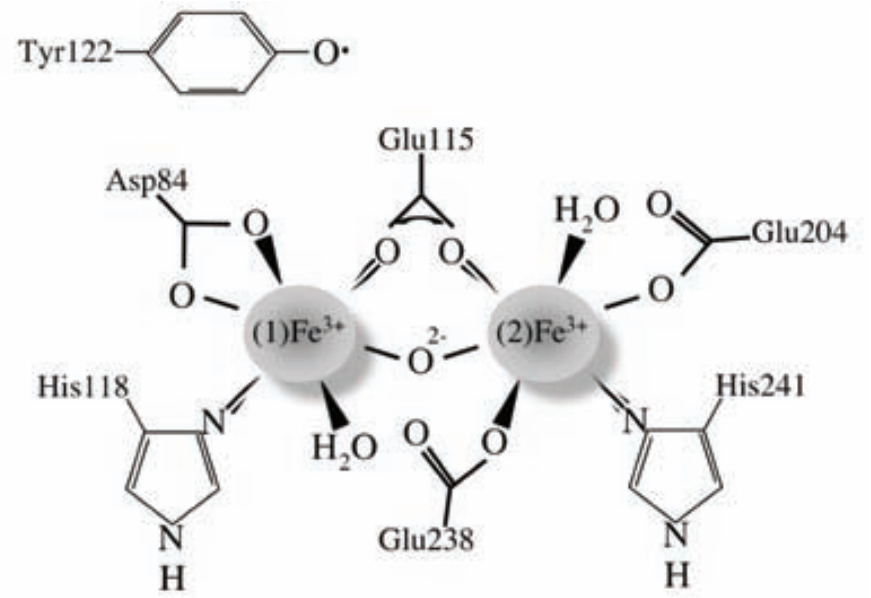

Figure 8. Schematic representation of the diferric center from active R2

It is the reduced form of the iron center of class I RNR that is responsible for activating molecular oxygen in the radical generation reaction. Initially, two ferrous ions bind to the apo protein to generate the reduced form. Once this reduced form is exposed to air, both the iron center and Tyr122 will be simultaneously oxidized by molecular oxygen to give active R2 (Sahlin *et al.*, 1990).

The overall reaction can be drawn as follows:

$$2Fe^{2+}+Tyr\text{-}OH+O_2+H^{+}+e^{-} \longrightarrow Tyr\text{-}O\bullet/Fe^{3+}\text{---}O\text{---}Fe^{3+}+H_2O$$

The generation of the diiron-Y• cofactor requires Fe^{2+} binding and a four-electron reduction of O_2 to H_2O, as well as the interaction of R2 with an iron delivery system and a protein such as a flavin reductase to deliver the reducing equivalent (Eliasson *et al.*, 1986; Fontecave *et al.*, 1987; Fontecave *et al.*, 1989). Three of the electrons required for O_2 reduction are provided by the oxidation of iron(II) to iron(III) and from the oxidation of Tyr122 (thus generating the tyrosyl radical), but the source of the fourth electron *in vivo* has not been identified yet (Elgren *et al.*, 1991; Ochiai *et al.*, 1990). Some studies by Stubbe, Bollinger and co-workers suggested a key role for Trp48 in a reversible electron transfer pathway between Trp48 and Tyr122, providing the fourth electron needed for the complete reduction of dioxygen (Bollinger *et al.*, 1995; Saleh & Bollinger, 2006; Tong *et al.*, 1998). The tyrosyl radical in class Ia RNRs is characterized by a sharp absorbance at 410 nm (ε = 3900 M^{-1} cm^{-1}) and a doublet EPR (Electron Paramagnetic Resonance) spectrum (g = 2.0047) with superposition of additional hyperfine interactions.

The structure of the mammalian class Ia small subunit with fully occupied metal sites has also been solved from mouse and there are a few differences with the *E. coli* R2 protein that are worth noticing (Mann *et al.*, 1991; Nyholm *et al.*, 1993; Schmidt *et al.*, 1996; Strand *et al.*, 2004): (i) The metal centers of the mouse R2 enzyme are more labile. A continuous supply of ferrous iron and oxygen is needed to keep the enzyme fully active, while the metallosites and the tyrosyl radical of *E. coli* R2 are much more stable at physiological conditions and are capable of performing multiple redox cycles. (ii) The tyrosyl radical and the diiron site in mouse R2 are accessible to solvent through a narrow hydrophobic channel, but an aromatic ring blocks this channel in *E. coli*. (iii) The four carboxylates ligands of the iron center in mouse show a greater flexibility compared to *E. coli* R2. This greater flexibility has been suggested to contribute to the lower kinetic and thermodynamic affinity for iron(II) of the mouse R2 enzyme. (iv) The mouse R2 has a strong cooperative formation of the two binuclear ferrous centers, which could indicate a tighter regulated process for activating the mouse R2 protein.

1.5.1.2 - Class Ib

As for the class Ib R2F, the iron coordination in the *S. typhimurium* R2F protein is similar (although not identical) to that of class Ia *E. coli* R2 (Eriksson *et al.*, 1998). The iron atoms of the ferric R2F are coordinated by the same residues as in class Ia and they are also bridged by one of the glutamate residues and an O^{2-} ion. The coordination of Fe2 is identical to that in the *E. coli* R2. The coordination of Fe1 differs since it has a monodentate carboxilate binding of Asp84 (*E. coli* numbering), a water molecule shared with Fe2 and one additional water molecule. The overall arrangement is similar to that of the mouse R2. There are also some differences in the position of the tyrosine residue housing the radical. In the *S. typhimurium* R2F protein, the tyrosyl radical is located at a slightly greater distance from the closest iron than it is in the *E. coli* enzyme, and the environment that surrounds it is more hydrophobic. These differences are responsible for the characteristic and distinct EPR signal observed in all class Ib R2F subunits.

1.5.1.3 - Non-standard Class I RNRs

The recent sequencing of bacterial genomes has revealed the presence, in all *Mycoplasma* species and in three *Streptococcal* species (*S. pyogenes*, *S. agalactiae* and *S. equi*) of an unusual class Ib R2F protein where three out of the six conserved iron-binding residues are missing (Chambaud *et al.*, 2001; Ferretti *et al.*, 2001; Fraser *et al.*, 1995; Himmelreich *et al.*, 1996). Up to date, however, there are no studies confirming the actual role of these proteins in ribonucleotide reduction. Since the genus *Mycoplasma* comprises a group of parasitic bacteria, it is possible that these parasites cover their need for dNTPs by taking them up directly from the infected host organism, and the three *Streptococcal* species mentioned above harbour a second R2F protein containing all the conserved residues for proper radical generation.

The Y4 (β subunit) protein of *S. cerevisiae* also has substitutions in three out of the six iron binding residues and it is essential for growth under some genetic backgrounds, forming and heterodimeric R2 subunit together with the normal β subunit that is also present in yeast (Ge *et al.*, 2001) (see below).

The class I RNR of *Corynebacterium ammoniagenes* was initially thought to be manganese-dependent (instead of iron-dependent) and, hence, it was reallocated as a class IV RNR (Oehlmann & Auling, 1999), but subsequent experiments from Sjöberg and co-workers demonstrated that it was indeed a diferric protein belonging to class Ib RNRs (Huque *et al.*, 2000).

The class Ib RNR from the Gram-positive bacterium *Bacillus anthracis* contains and NrdF subunit that shows an EPR spectrum slightly different from that of previously reported class Ib enzymes (Torrents *et al.*, 2005b). The three-dimensional geometry of its radical side chain shows a 60º rotational angle of the aromatic ring which differs from the typical 75-85º rotational angle found for class Ib radicals, suggesting a different geometry of its diiron-radical site. Additionally, the authors showed a different relaxation behaviour of its tyrosyl radical which could indicate a weaker interaction between the radical and the diiron site. The *Bacillus subtilis* NrdF protein was also proven more resistant to the radical scavenger hydroxyurea (see section 1.11) compared with the *E. coli* enzyme, and extremely more sensitive to the antioxidants hydroxylamine and N-methyl hydroxylamine (see section 1.11). This unusual behaviour may be due to the unique properties of its diiron-radical site.

Another non-standard group of class Ia RNRs is found among some human pathogens, such as *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Tropheryma whipplei* (Bentley *et al.*, 2003; Kalman *et al.*, 1999; Stephens *et al.*, 1998) and also in the archaean *Halobacterium* sp. NRC-1 (Ng *et al.*, 2000). In this group of microorganisms the essential tyrosine radical is substituted with phenylalanine, although the enzyme purified from *C. trachomatis* has proven active, suggesting an alternative route for the generation of the thiyl radical at the active site in R1 (Hogbom *et al.*, 2004). It seems that the activity of the chlamydial RNR relies on a FeIII-FeIV iron site in R2. This site replaces the normal tyrosyl radical and participates in the subsequent radical formation in R1 upon substrate binding (Voevodskaya *et al.*, 2006).

1.5.2 - Class II RNRs

Class II RNRs, in contrast to class I RNRs, do not have a separate protein radical site, instead, they generate a cysteine radical directly at their active site by using the B_{12}

derivative 5'-deoxyadenosylcobalamin (Booker *et al.*, 1994). The thiyl radical is generated after the homolytic cleavage of the deoxyadenosylcobalamin bond generating cobalt(II) and a deoxyadenosyl radical that probably abstracts a hydrogen atom from Cys408, which yields the S• (Fig. 9).

The AdoCbl cofactor replaces the role of the β subunits of class I and III RNRs as the radical generator and thus, this class does not contain any metallosite. It is believed that binding of the B_{12} cofactor induces a conformational change into a more "closed" conformation, which seems to be essential to protect the highly reactive nucleotide radical intermediates from oxygen and solvent, and for the proper positioning of B_{12} relative to Cys408 (Sintchak *et al.*, 2002).

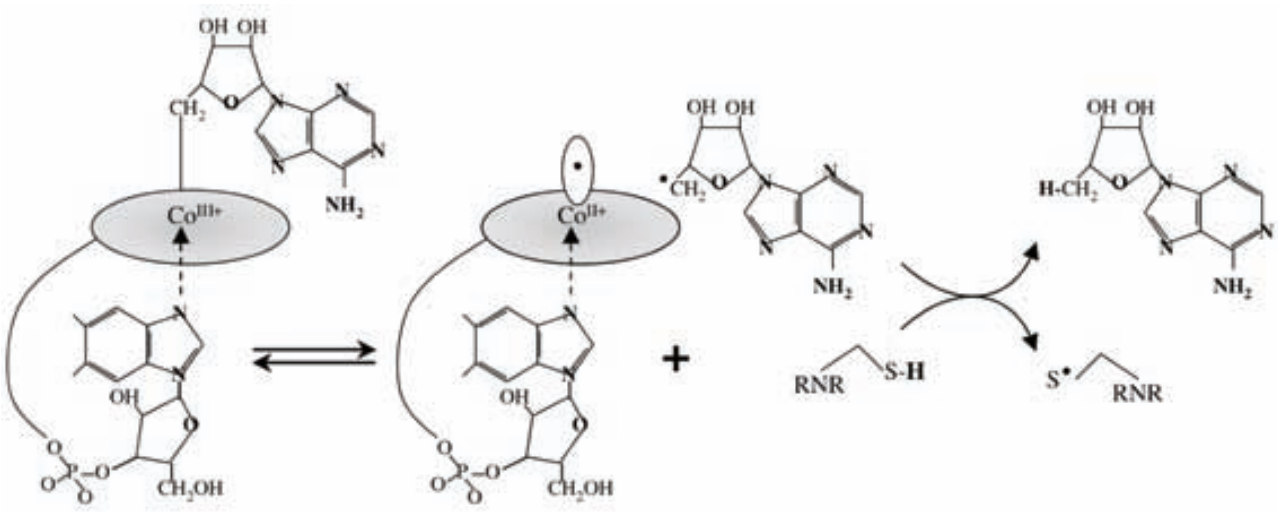

Figure 9. Thiyl radical generation in class II RNRs. Addition of the effector enhances homolysis of the Co-5´CH2 bond, generating an adenosyl radical that will abstract the hydrogen from the active site cysteine. (Adapted from Ludwig & Matthews, 2002)

1.5.3 - Class III RNRs

Class III RNRs contain a stable glycyl radical near the C-terminus of the α subunit (NrdD), which also harbours the active and the allosteric regulatory sites. The α_2 dimer, therefore, constitutes the active enzyme, but needs to be activated by a β subunit (NrdG),

called the activase, which carries out the homolytic cleavage of S-adenosylmethionine (SAM, AdoMet) in association with an iron-sulfur cluster ([4Fe-4S]) to generate the stable glycyl radical of NrdD (Ollagnier *et al.*, 1997). Generation of this glycyl radical comprises three main steps (Fig 10): (i) Reduction of the [4Fe-4S] cluster by a flavodoxin/flavodoxin reductase system (Bianchi *et al.*, 1993). (ii) Reduction and cleavage of AdoMet, probably by direct electron transfer from the cluster, and formation of a 5'-deoxyadenosyl radical along with methionine (Ollagnier *et al.*, 1997). (iii) Generation of the glycyl radical by the 5'-deoxyadenosyl radical through abstraction of a hydrogen atom from the Cα of the glycine (Gly580 in T4) (Gambarelli *et al.*, 2005). Upon exposure to oxygen, class III RNRs undergo a polypeptide chain cleavage at the glycyl radical position, which results in inactivation of the enzyme (King & Reichard, 1995).

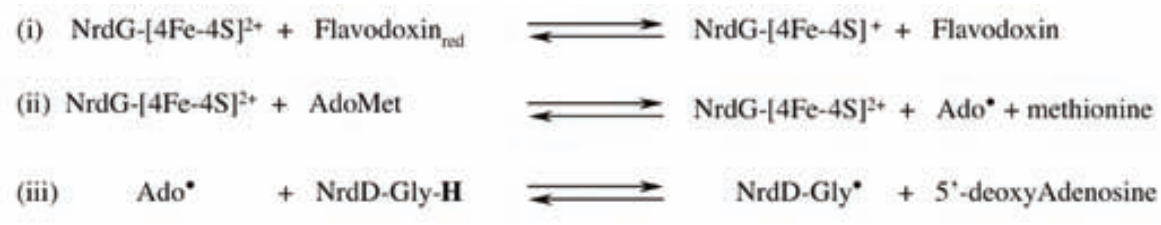

Figure 10. Proposed mechanism for the generation of the glycyl radical in class III RNRs. (Taken from Mulliez *et al.*, 2001)

1.6 - Radical Transfer Reaction

In class I RNRs, the crystal structure of the R2 protein shows that the tyrosyl radical is buried inside the protein about 10 Å from the closest surface (Nordlund *et al.*, 1990). This radical, however, is used to generate a transient thiyl radical at the active site of R1. At first it was believed that the tyrosyl radical in R2 and the active site from R1 could directly interact through considerable conformational changes in the proteins. However, once it was proven that the spectroscopic characteristics of the tyrosyl radical remained unchanged in the holoenzyme complex, as compared with the R2 protein alone, this hypothesis was rapidly ruled out (Sahlin *et al.*, 1987). If the radical could not interact directly with the active site, an

indirect mechanism had to be present. Even under the most favourable structure, the radical has to travel over a distance of 30-40 Å.

Back in 1994, the only picture of the R1-R2 holoenzyme complex consisted of a docking model composed of the crystal structures of R1 and R2 (Uhlin & Eklund, 1994), and it was used to propose a fascinating long radical transfer chain to connect the essential tyrosyl radical in R2 and the putative thiyl radical at the active site of R1 (Fig. 11).

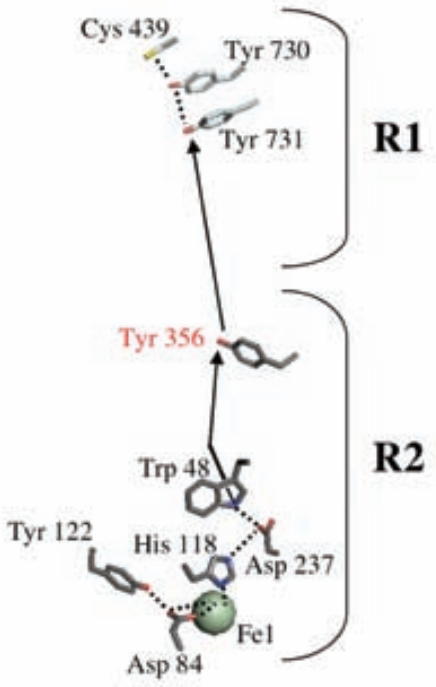

Figure 11. Proposed long-range proton coupled electron transfer between the tyrosyl radical in R2 and Cys439 in the active site of R1. (Adapted from Sjöberg, 1997)

The radical was proposed to be transferred by proton coupled electron transfer via a chain of hydrogen bonded aminoacid side chains (Nordlund *et al.*, 1990; Stubbe *et al.*, 2003). A number of conserved residues forming a pathway of hydrogen bonds that connects both the tyrosyl radical site, and the thiyl radical site, have been found by comparing several class I RNRs sequences, and site directed mutagenesis experiments have confirmed their role in radical transfer (Ekberg *et al.*, 1996; Ekberg *et al.*, 1998; Rova *et al.*, 1995; Rova *et al.*, 1999). These residues are (*E. coli* numbering): Cys439, Tyr730, and Tyr731 in R1, and Asp84, His118, Asp237, Trp48, and Tyr365 in R2. In R2, the Fe1 of the diiron cluster is also

included. It seems that the enzyme exerts a strict control over the release of the oxidizing power from the free radical at Tyr122 to ensure that only when the substrate is correctly bound, the radical transfer process is initiated. This safety mechanism avoids harmful side reactions.

In class II RNRs there is no need for a radical transfer reaction, since the thiyl radical is directly generated by the homolytic cleavage of the cobaltous cofactor.

A similar situation is found in class III RNRs, where the glycyl radical is located within the same subunit as the cysteine radical at the active site. The glycine housing the radical is at the tip of a loop that inserts into the active site so that the distance between the glycyl site and the central cysteine is about 5 Å. It is then feasible to transfer the glycyl radical to the closely located cysteine, either by direct abstraction or via neighbouring residues, in contrast to the long radical transfer reaction described for class I RNRs (Logan *et al.*, 1999) (Fig. 12).

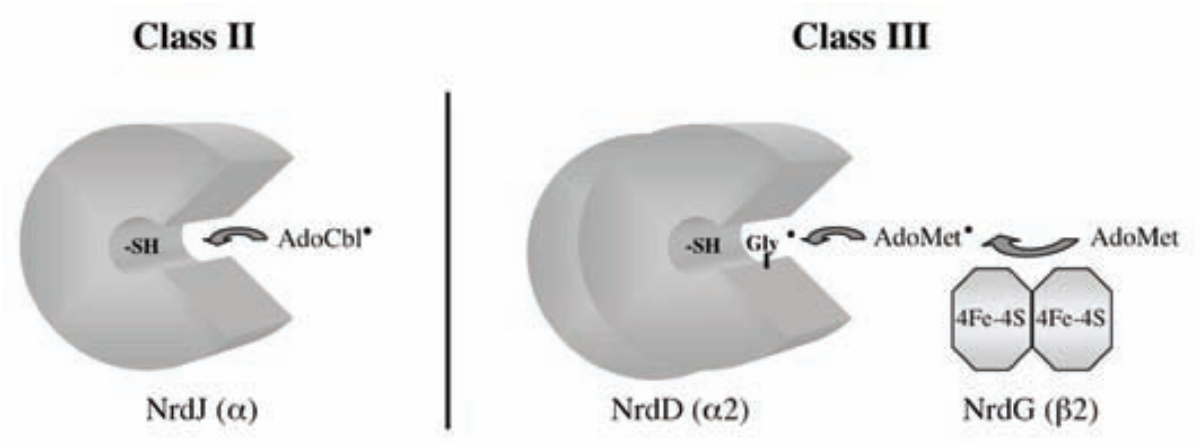

Figure 12. Schematic model representing the radical transfer reaction in class II and class III RNRs

1.7 - Reaction Mechanism

Despite their structural and allosteric differences, all three RNR classes seem to share a similar reaction mechanism to perform the reduction of the hydroxyl group at the 2'-position of a di- or triphosphate ribonucleoside. The general mechanism was proposed by Stubbe,

mainly on the basis of experiments with mechanism-based inhibitors and site directed mutants (Mao *et al.*, 1992; Stubbe, 1990). Although most of these studies have been performed on class I enzymes and, thus, the *E. coli* class Ia enzyme will be used as a model in the following discussion, there are structural and biochemical evidences that support a common mechanism in all three classes (see (Booker *et al.*, 1994; Lawrence & Stubbe, 1998; Sintchak *et al.*, 2002) for class II RNRs and (Eliasson *et al.*, 1994a; Fontecave *et al.*, 2002; Logan *et al.*, 1999) for class III enzymes).

Ribonucleotide reduction by means of radical chemistry has in common the use of highly conserved active site cysteines. The active site of R1 contains three cysteine residues essential for substrate reduction: two reducing cysteines (Cys225 and Cys462) and Cys439, which houses the transient thiyl radical required to initiate the reaction.

The reaction is initiated upon binding of the substrate to the active site (Fig. 13); **(1)** the thiyl radical at Cys439 abstracts the 3'-hydrogen atom from the ribose ring of the substrate and thereby generates a transient radical on the ribose. **(2)** Cleavage of the 2'-C-O bond and protonation of the leaving water molecule generates a radical on the proximal reducing cysteine (Cys225). **(3-4)** Deprotonation of the 3'-OH group promotes formation of a disulfide anion radical and protonation of the 2'-carbon. **(5)** The excess radical electron is then transferred via a chain of hydrogen bonded active site residues, Asn437 and Glu441, to the substrate 3'-position, regenerating a substrate radical and forming a disulfide bridge between the cysteines. **(6)** This substrate radical abstracts the stored hydrogen atom from Cys439 regenerating a thiyl radical on Cys439 and releasing a deoxyribonucleotide product. The radical state is then transferred back to Tyr122 in the R2 subunit via the long radical transfer chain, regenerating the stable Tyr122•.

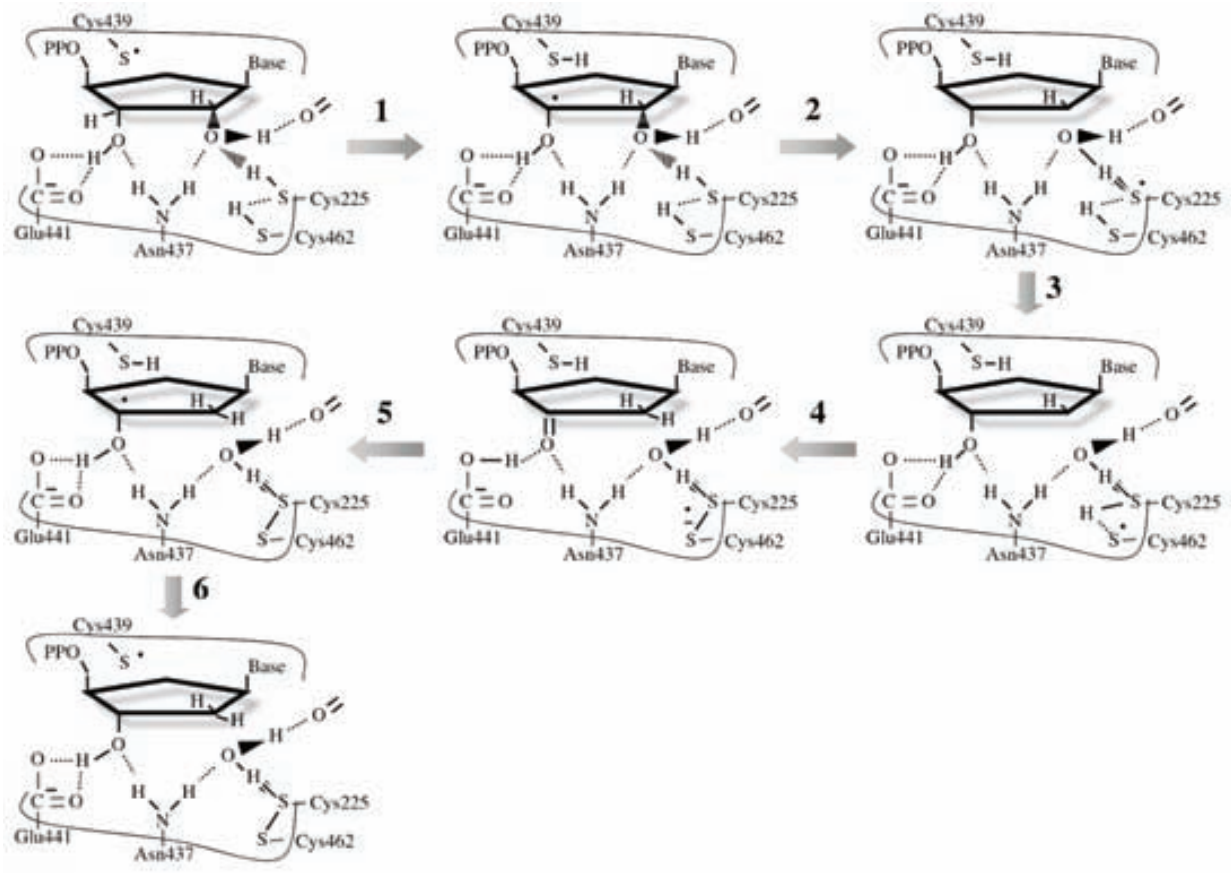

Figure 13. Proposed reaction mechanism for class I and II ribonucleotide reductases. (Adapted from Nordlund & Reichard, 2006)

The active site of class II RNRs contains all the conserved residues found in class I RNRs; the cysteine radical site Cys439 in *E. coli* class Ia is located in Cys408 in *L. leichmannii*, and the essential residues Asn437, Glu441, Cys225 and Cys462, described for *E. coli*, have their equals in Asn406, Glu410, Cys119 and Cys419 in *L. leichmannii*, indicating a very similar reaction mechanism. Furthermore, the pair of additional cysteines that shuttle electrons from the hydrogen donor systems to regenerate the active site in class I RNR are also conserved in *L. leichmannii*.

The structure of class III RNR from bacteriophage T4, however, shows only two highly conserved cysteines at its active site, Cys290 and Cys79. Cys290 is thought to harbour the thiyl radical and it is in the same position as Cys439 in class Ia and Cys408 in class II RNRs. Cys79 is located at a similar position as Cys225 in class Ia RNRs and Cys119 in the *L.*

leichmannii enzyme, but class III RNRs lack a redox partner for this cysteine residue. The essential glutamate and asparagine residues found in class I and class II are also missing. In class III RNRs, the lack of both a reducing pair of cysteines and the essential glutamate and asparagine residues suggests that some steps of the reaction mechanism must be slightly different from those of class I and class II RNRs, although many other features of the reaction mechanism might be similar since many mechanism based inhibitors of class I and class II enzymes are also good inhibitors of class III RNRs (Eliasson *et al.*, 1994a). It is likely that formate acts as a proton donor in the initial steps of the reaction (instead of Glu441) and as a hydrogen donor at a later stage (replacing Cys462).

1.8 - Structure

1.8.1 - Class I

1.8.1.1 - R1 from *E. coli*

As mentioned above, R1 is composed of two α subunits (NrdA), each containing an active site and two allosteric binding sites with a molecular weight of 86.000 Da and 761 residues/monomer. The R1 dimer is S-shaped and the three-dimensional structure of each monomer can be divided in three domains (Uhlin & Eklund, 1994): a ten-stranded β/α barrel domain of approximately 480 residues, a mainly helical domain comprising the 220 N-terminal residues, and a small αβααβ domain comprising 70 residues (Fig. 14). The active site with Cys225, Cys 439 and Cys 462 is located deep inside a cleft between the N-terminal and barrel domains. The dimer is mainly stabilized by a four-helix bundle formed by two helices of the β/α barrel of each subunit, and the interactions between the subunits are predominantly van der Waal's contacts between the residues of these four helices, although a few hydrogen bonds are also formed (Larsson Birgander *et al.*, 2005).

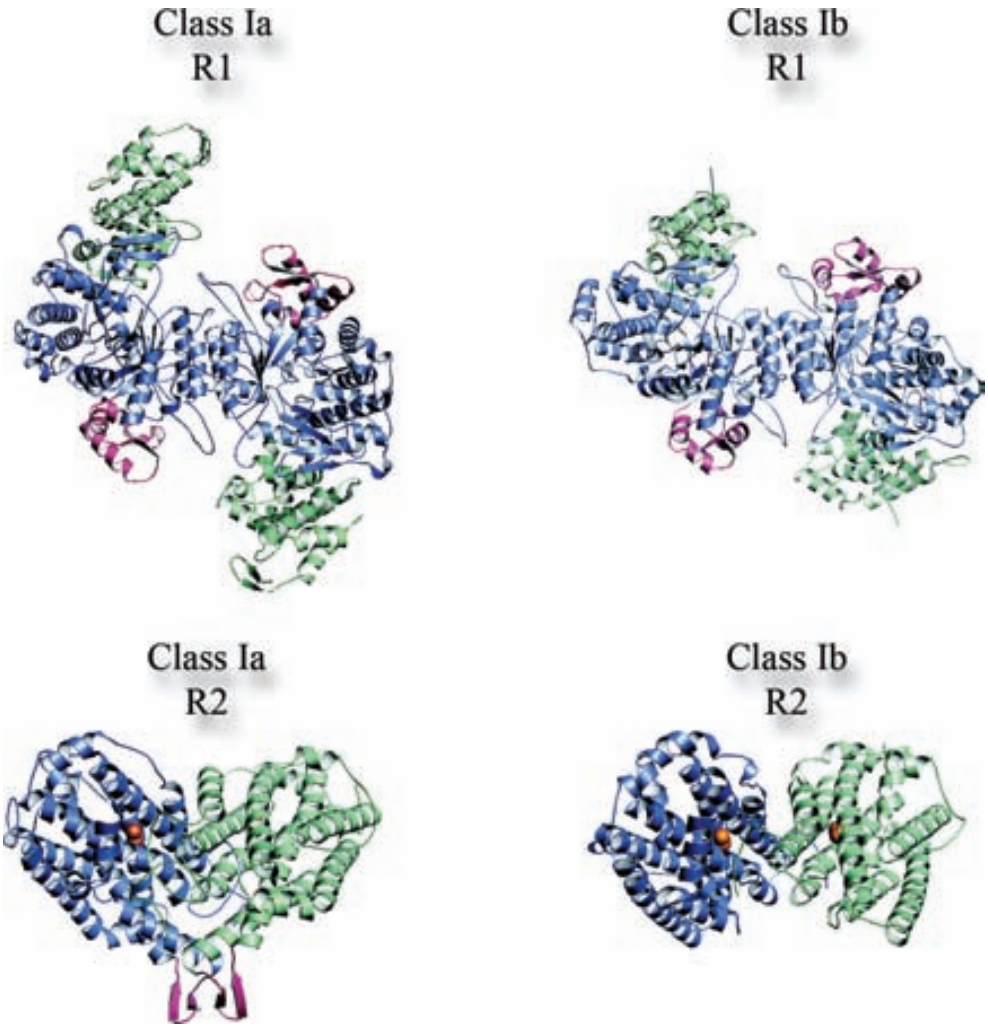

Figure 14. Comparison of the three-dimensional structures of the large and small subunits of class Ia (*E. coli*) and class Ib (*S. typhimurium*) RNRs. In R1, The N-terminal domains are shown in green. The conserved β/α barrel domains are shown in blue and the small αβααβ excursions are shown in purple. In the homodimeric R2 structures, one subunit is colored in blue and the other subunit in green. The beta-pleated sheet that constitutes the tip of the heart in the class Ia R2 subunit is shown in red. The iron atoms are shown as orange spheres

When the structure of the R1 protein from *E. coli* was first resolved, about 10% of all enzyme structures solved contained a TIM barrel-like domain, which consisted of eight consecutive βα units with the parallel strands in the center of the barrel and the helices on the outside. The ten-stranded barrel found in R1 constituted a novel type of barrel that contained a central pleated sheet of ten β-strands, instead of eight.

Moreover, the R1 barrel is larger than the common TIM barrels and has the β-strands differently arranged. It is composed of two halves connected in an antiparallel fashion, each containing five strands and four connecting helices (Uhlin & Eklund, 1996). The hydrogen bonding within each half is parallel, whereas the hydrogen bonding between the first and the last strand of each half-barrel is antiparallel. An appealing property of this particular domain is that the wider ten-stranded β/α barrel allows the insertion of a β-hairpin loop in its center. The essential active site residues can be found at the tip of this loop, deep inside the center of the barrel, particularly Cys439, which initiates the reaction mechanism upon generation of the transient thiyl radical. The tip of the finger loop also contains the conserved Asn437 and Glu441. The redox active cysteines Cys225 and Cys462 are located at equivalent positions on the first strand of each half-barrel. The arrangement of Cys439 at the tip of the loop is essential to ensure that the transient highly reactive thiyl radical only reacts with the substrate, since the side-chain of Cys439 is not in contact with any residue except Tyr730, which is involved in the radical transfer reaction from R2 to generate the thiyl radical (Fig. 15).

The active site in R1 is arranged in such a way that the redox-active pair of cysteines are facing the 2' and 3'-OH groups of the ribose ring, while Cys439 remains on the other side of the substrate, ready to abstract the 3'-hydrogen atom. The unique structure of the ten-stranded β/α barrel with an internal loop allows each important residue to perform its task without interfering with each other. The three cysteines are located on the inside of the top surface of the barrel at about 15 Å from the entrance to the cleft. The entrance to the active site is formed by the shorter strands in the first half-barrel, while the long strands in the second half-barrel form a wall on the inside of the active site.

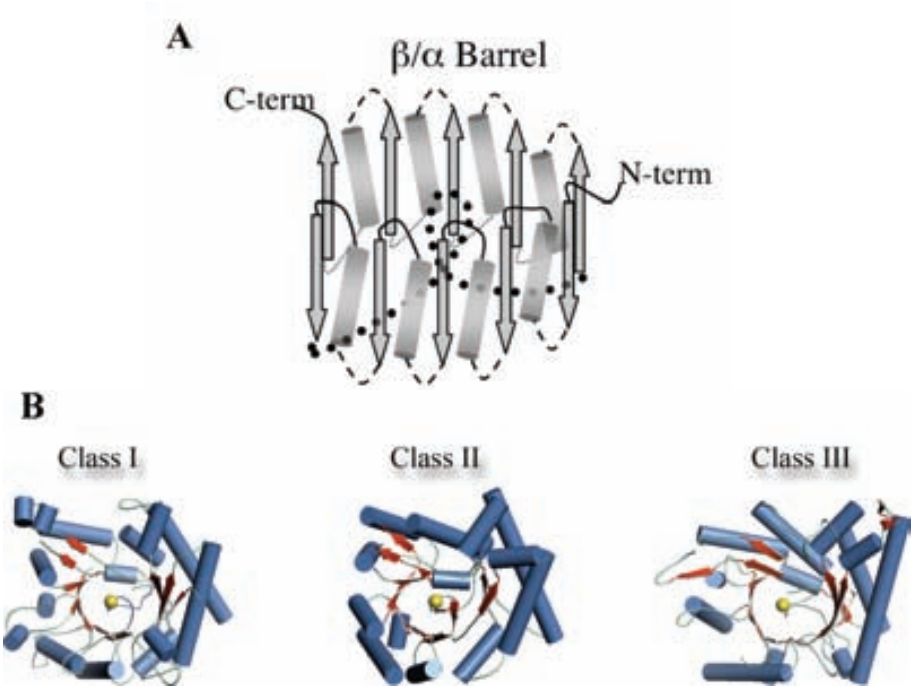

Figure 15. A) Schematic model of the ten-stranded β/α barrel. B) Comparison of the conserved ten-stranded β/α barrels from class Ia, class II and class III RNRs. The internal "finger-loop" housing the active site cysteine at its tip (yellow sphere) is also shown. (Adapted from Sintchak *et al.*, 2002)

The N-terminal domain constitutes a cover over the active site side of the barrel. It contains a pleated sheet followed by a four-helix bundle, five small helices and a β-hairpin. The N-terminal domain is attached to the ten-stranded barrel by hydrogen bonds to the long first strand of the second half-barrel. The allosteric activity site is located at the tip of the N-terminal domain, where the first 100 residues form a cleft with the four-helix bundle covered by a three-stranded mixed β-sheet, constituting an ATP-cone (Aravind *et al.*, 2000) with very conserved residues involved in ATP/dATP binding, as shown in complexes with the allosteric effector analogue AMPPNP (Eriksson *et al.*, 1997). The N-terminal tip of R1 is far away from the active site and the allosteric specificity site, but it is close to the proposed interaction area with R2 (Uhlin & Eklund, 1994). Recent studies have shown that, upon dATP binding, the allosteric activity site probably influences the overall activity of the enzyme by modulating the formation of tighter R1-R2 complexes that are unfavourable for ribonucleotide reduction (Kasrayan *et al.*, 2004; Larsson *et al.*, 2004b).

The substrate specificity site has been characterized by using R1 crystals soaked with dTTP (Eriksson *et al.*, 1997) and it has been shown that the nucleotide effectors bind at the

end of a four-helix bundle formed across the dimer interface. Since the distance between the substrate specificity site and the closest active site is too far to establish direct interactions between the effector and the substrate (approximately 15 Å), the effect of the allosteric effectors is probably due to protein interactions.

The αβααβ small domain forms an excursion from the ten-stranded β/α barrel and it contains many conserved residues, a majority of which are hydrophobic and participate in packing against the barrel domain.

1.8.1.2 - R1E from *S. typhimurium*

The structure of the R1E subunit of class Ib from *Salmonella typhimurium* (NrdE) has been recently determined together with its allosteric effectors (Uppsten *et al.*, 2003). The R1E structure is very similar to the *E. coli* R1 protein and it contains a ten-stranded β/α barrel domain of 480 residues, a small α+β domain at the bottom of the barrel and a short N-terminal domain. The N-terminal domain, however, lacks approximately 50 amino-terminal residues that in the *E. coli* subunit form the overall activity site (Fig. 14). The lack of these N-terminal residues implies the loss of dATP inhibition and it is a common feature of all R1E proteins, constituting the major structural difference between class Ia and class Ib RNRs. Some minor differences are found in the exact conformation and length of some loops within the barrel domain as well as in the most exposed parts of the small domain, but the overall three-dimensional structure of R1 and R1E are very much alike.

1.8.1.3 - R2 from *E. coli*

The *E. coli* R2 was the first RNR structure to be solved (Nordlund *et al.*, 1990; Nordlund & Eklund, 1993). It contains the organic free radical at Tyr122 and a dinuclear iron center. It is composed of two β subunits (NrdB) with a molecular weight of 43,500 Da and 375 residues each. Each subunit constitutes a single domain of 13 helices and two β-sheets strands, which has been described as three layers of helices. The first layer establishes most of the subunit-subunit contacts that contribute to dimer formation, but the first β-pleated sheet strand and the random coil formed by the first 33 N-terminal residues also participate in the maintenance of the dimer. The R2 dimer resembles the shape of a heart, since the helices at

the dimer interface cross at an angle of about 140º to each other and the pair of β-hairpins from each subunit form a four-stranded antiparallel pleated sheet that draws the pointed tip of the heart (Fig. 14).

The C-terminal part of R2 has been shown to participate in the R1-R2 holoenzyme formation. The C-terminal 35 residues form an ordered tail not visible in the electron density maps that probably becomes ordered when binding to the R1 protein. Peptides and peptidomimetics corresponding to the R2 protein C-terminal tail inhibit ribonucleotide reductase in a species-specific way.

The main function of R2 is to house the stable radical and transfer it to the large subunit upon substrate binding. The structure and location of both the diiron center and the tyrosyl radical have been described previously and will not be discussed on this section.

The crystal structure of the mouse R2 enzyme has also been solved and shown to be an all-helical protein (Kauppi *et al.*, 1996). Overall, it resembles the structure of the R2 protein from *E. coli* although it lacks the two β-sheet strands, a feature that shares with the R2 subunits from class Ib enzymes (see below).

1.8.1.4 - R2F from *S. typhimurium*

The three-dimensional structure of the R2F protein from *Salmonella typhimurium* (NrdF) was solved by Eriksson and co-workers in 1998 (Eriksson *et al.*, 1998) and it has an overall structure similar to that of the *E. coli* R2 protein. However, it differs from the class Ia subunit in the absence of the β-pleated sheet that in the *E. coli* protein constitutes the pointed tip of the heart (Fig. 14). The lack of the two β-sheet strands has also been described for the solved structures of the R2F proteins from *C. ammoniagenes* (Hogbom *et al.*, 2002) and *M. tuberculosis* (Uppsten *et al.*, 2004) and seems to be a common feature to all class Ib enzymes. Further differences concerning the metal sites and the tyrosyl radical have been previously mentioned.

1.8.1.5 - The R1-R2 holoenzyme

For many years, the accepted structure of the R1-R2 holoenzyme was based on a symmetric docking model proposed by Uhlin and Eklund (Uhlin & Eklund, 1994), which

used the structures of the two dimeric R1 and R2 subunits. In this model, R1 was suggested to sit on top of the heart-shaped R2 since the shape of the R1 dimer was complementary to the upper part of the heart. The modelled holoenzyme was more compact than the R1 dimer, suggesting a much more stable structure. There were several reasons that made this model plausible: (i) The docking model positioned the tyrosyl radical in R2 and the active site cysteines in R1 at a distance not greater than 40 Å, the shorter distance provided by any modelled structure of the holoenzyme. (ii) In R1, a conserved hydrogen bonded pathway could be drawn from the active site Cys439 to Tyr731 at the interaction area with R2, where a second conserved hydrogen bonded pathway connected Trp48 at the top surface of R2 with the buried tyrosyl radical at Tyr122. (iii) The C-terminal tail of R2, known to be involved in the R1-R2 interaction, localized at the top surface of R2. (iv) The model strengthened the experimental evidences showing that effector binding influenced the nature of R1-R2 interactions, since the allosteric specificity sites were placed close to the interaction area.

It was thought that formation of the R1-R2 holoenzyme was driven by both hydrophobic and electrostatic interactions, since a mixture of conserved hydrophobic and hydrophilic side chains were found at the proposed interaction surfaces of R1 and R2.

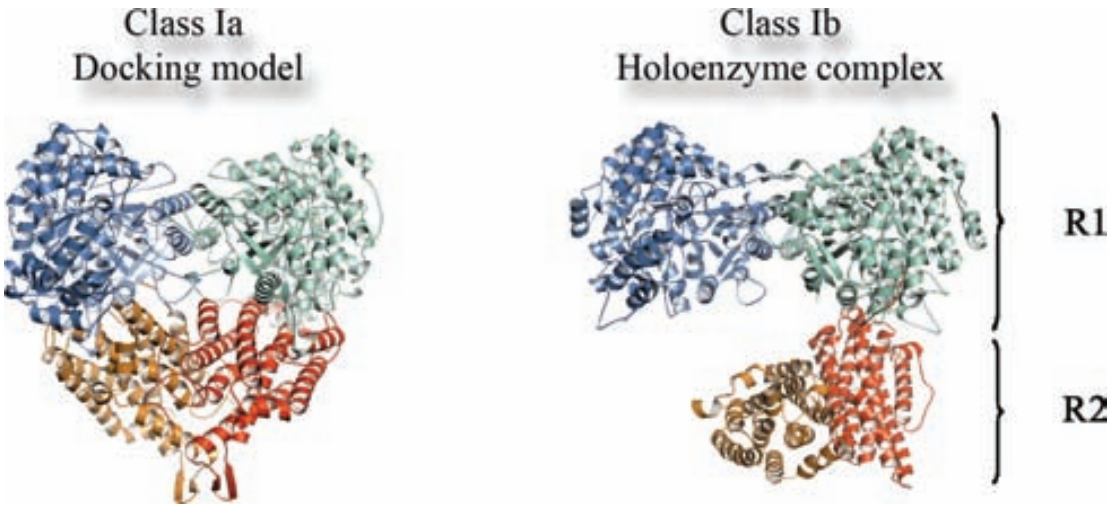

Figure 16. Three-dimensional docking model of the class Ia R1-R2 complex from *E.coli* (left). Three-dimensional structure of the class Ib RNR holoenzyme complex from *S. typhimurium* (right). R1 monomers are shown in blue and green and R2 monomers in red and brown. (Adapted from Uppsten *et al.*, 2006)

In 2006, the first crystal structure of the *S. typhimurium* class Ib R1-R2 holoenzyme was finally published by Uhlin and co-workers (Uppsten *et al.*, 2006). The complex structure confirmed that the C-terminal tail of R2F constitutes the linking bridge between both dimers, binding in a hydrophobic cleft between three α helices from R1E. The crystal structure, however, was shown to be asymmetric, with only one R2F monomer interacting with R1E, while the other monomer hung from its dimer partner (Fig. 16). The authors proposed that such asymmetric R1-R2 complex represented an intermediate stage towards formation of an active holoenzyme, and it could be used to explain the half-site reactivity observed in many RNR enzymes, where only one tyrosyl radical per dimer was detected (Sjoberg *et al.*, 1987). Unfortunately, the asymmetric nature of this complex, as well as the low resolution achieved (4 Å), provided little evidence to corroborate the radical transfer pathway theory. The authors suggested that binding of the substrate at the active site of R1 might promote a tightening of the complex and formation of a short-lived symmetric holoenzyme which, in turn, would facilitate the proper positioning of the subunits to allow radical transfer. Under this model, further reduction of the substrate would cause the holoenzyme to rapidly open up. The opening of the complex might result in the C-terminal tail of R2F attached to the other R1 monomer, suggesting that the active sites take turns in each reducing cycle.

1.8.2 - Class II

The crystal structure of the class II ribonucleoside triphosphate reductase from *Lactobacillus leichmannii* (NrdJ) was finally solved by Drennan and co-workers (Sintchak *et al.*, 2002) and it provided the missing piece to complete the collection of solved RNR structures. The monomeric class II RNR from *L. leichmannii* has a molecular weight of approximately 82,000 Da and 738 residues arranged in a global fold composed of a ten stranded β/α barrel very similar to those described for class I and class III (see below) RNRs (Fig. 15). A conserved finger loop can also be found at the center of the barrel, with the active site thiyl radical located at the “fingertip” residue Cys408. Most of the key active site residues in class I and class III are also structurally conserved.

Surprisingly, and despite their different substrate preferences, there are remarkable similarities between the substrate-binding region of the *L. leichmannii* class II RNR and the *E.*

coli R1 subunit (the *L. leichmannii* enzyme binds nucleoside triphosphates (NTPs) while the *E. coli* class I enzyme prefers nucleoside diphosphates (NDPs)). The only significant difference that might explain their different substrate preferences is the binding of B_{12} in the vicinity of the substrate-binding region of the *L. leichmannii* enzyme, although it needs further investigation. The B_{12} binding region of the *L. leichmannii* class II enzyme is structurally different from other known B_{12}-binding domains and, thus, it uses a newly discovered and unique fold relative to other B_{12}-dependent enzymes. B_{12} binding apparently induces a conformational change into a more "closed" conformation, which seems to be essential to protect the highly reactive nucleotide radical intermediates from oxygen and solvent, and for the proper positioning of B_{12} relative to Cys408, allowing the homolytic cleavage of the cobaltous cofactor and formation of the transient thiyl radical (Fig. 17).

The substrate specificity site of the *L. leichmannii* class II RNR is, by far, its most appealing characteristic due to the monomeric nature of this enzyme. The four-helix bundle formed at the dimer interface in the *E. coli* R1 subunit is mimicked in the *L. leichmannii* protein by two helices from the β/α barrel in combination with an extra fragment of 130 residues that inserts into the barrel domain, preserving the dimer interface in a monomeric protein. In the dimeric class I RNRs, the allosteric sites influenced the activity of the enzyme either by modulating the association/dissociation of the R1-R2 complexes or by establishing an intermolecular communication, but in the monomeric class II RNR from *L. leichmannii* a more likely scenario would be that of direct linkage between the binding sites, so that effector binding may propagate intramolecular conformational changes to alter the binding specificity at the active site. Stubbe and co-workers have shown that the binding of effector alone can trigger homolysis of the AdoCbl and generation of the transient thiyl at Cys408 (Licht *et al.*, 1999). Note that being a class II RNR, the *L. leichmannii* enzyme lacks the allosteric activity site.

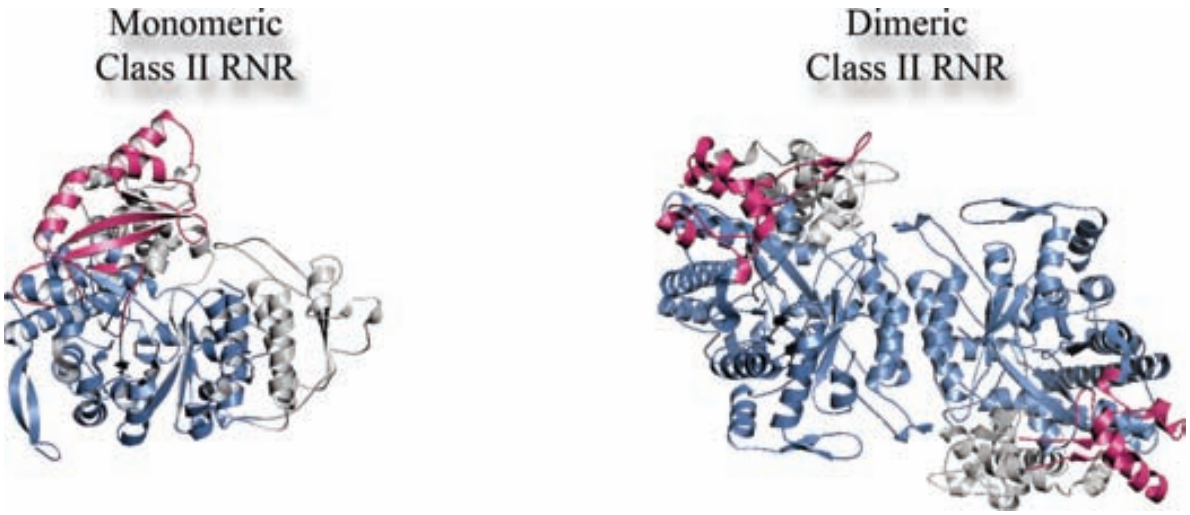

Figure 17. Comparison of the three-dimensiona structures of the monomeric (*L. leichmannii*) and dimeric (*T. maritima*) class II RNR. The conserved β/α barrel domains are shown in blue and the B12-binding regions are shown in purple. (Adapted from Nordlund & Reichard, 2006)

Overall, the structures of the *L. leichmannii* class II and the *E. coli* class Ia RNRs are very much alike, despite the low levels of similarity between their polypeptide sequences.

A recent study concerning the structure of the dimeric class II enzyme from *Thermotoga maritima* in complex with different specificity effectors provided further evidence supporting a common mechanism for allosteric specificity regulation (Larsson *et al.*, 2004a) (Fig. 17). Although the *T. maritima* enzyme lacks the 130-residues insertion described for the *L. leichmannii* monomeric class II, effector binding still takes place at a four-helix bundle now formed at the dimer interface, pretty much the same way it does in class Ia RNRs. Moreover, the high resolution complexes achieved in this study suggested a high degree of cooperativity between effector binding and substrate specificity through conformational changes that are conserved in all three classes.

1.8.3 - Class III

The only class III structure available up to date is that of the bacteriophage T4 large subunit (Logan *et al.*, 1999), which was carried out using a mutant where the glycyl radical

site was replaced by an alanine residue, to avoid aerobic cleavage of the polypeptide chain. Class III RNRs have a quaternary structure $\alpha_2\beta_2$ (Sun *et al.*, 1995; Young *et al.*, 1996). The large α subunit (NrdD) has a molecular weight of 80,000 Da and 712 residues and it contains the allosteric regulatory sites, the active site and the glycyl radical needed to initiate the reaction mechanism (Fig. 18). The β subunit (NrdG), also known as the "activase", is a member of the recently discovered "radical-SAM" enzyme superfamily (Sofia *et al.*, 2001).

Figure 18. Three-dimensional structure of the class III NrdD protein from bacteriophage T4. The N-terminal domain is shown in green. The conserved β/α barrel domain is shown in blue and the long-interacting helices are shown in purple

This family of enzymes is characterized by a [4Fe-4S] center used to bind, reduce and cleave S-adenosylmethionine (SAM) into methionine and a 5'-deoxyadenosyl radical. The class III RNR small activase has a molecular weight of about 17,500 Da and approximately 154 residues and it plays an important role in generating the stable glycyl radical near the C-terminal end of NrdD. Unluckily, the crystal structure of the activase is not yet available and very little is known about the mechanisms involved in the transfer of the radical between NrdG and NrdD.

As it has already been described for class II RNRs and despite the lack of similarities at the primary sequence level, the overall fold of the large NrdD subunit is based around the same ten-stranded β/α barrel observed in the R1 structure of *E. coli*, with two antiparallel

halves, each containing five parallel β-strands, flanked on the outside by helices. The center of the barrel contains two antiparallel β-strands forming the prototypical "finger" loop with the cysteine residue that harbours the active site thiyl radical at its tip. The second active site cysteine, Cys79, is located at a similar position as Cys225 in class Ia RNRs and Cys119 in the *L. leichmannii* enzyme. There are no redox partners for this cysteine residue in class III RNRs, since it is reduced by formate rather than by thioredoxins or glutaredoxins.

In 2003, Logan and co-workers identified a $M(Cys)_4$ metal-binding site at the C-terminal end of NrdD from bacteriophage T4, which either bound iron or (more likely) zinc. Mutation of the highly conserved cysteines forming this site in the *E. coli* enzyme showed that they participated in the generation of the glycyl radical but not in interaction between the large subunit and the activase. The authors suggested a structural role for this $M(Cys)_4$, although its involvement in electron transfer was not excluded either (Logan *et al.*, 2003).

One of the major differences between class I and class III structures can be found at the dimer interface area, where four longer helices replace two of the helices that form the four-helix bundle in R1. The helices at the dimer interface in class III are antiparallel and are tilted with respect to the strands of the barrel, such that the orientation of the monomers greatly differs from that of R1. This particular orientation of the dimer axis is likely to affect the molecular mechanisms that communicate the specificity site with the active site, which apparently differ from those of class I and class II RNRs.

In a similar way, the mechanism of overall activity regulation must be also different to that of class I, since class III RNRs are functionally dimeric (the NrdG protein acts as a catalytic activator (Torrents *et al.*, 2001)) and effector binding does not regulate formation of tighter or looser heterotetramers, as proposed for class Ia (Kasrayan *et al.*, 2004; Larsson *et al.*, 2004b).

The overall structure of the bacteriophage T4 class III RNR is strikingly similar to that of pyruvate formate lyase (PFL), an ancient enzyme involved in anaerobic fermentation (Knappe & Wagner, 1995). PFL is a dimeric protein containing a glycyl radical near its C-terminal end, and formation of the radical in PFL also requires the help of S-adenosylmethionine plus an iron-sulfur cluster on an activating protein, denoted PFL activase. Furthermore, the three-dimensional structure of PFL contains a ten-stranded β/α barrel with two important cysteine residues located on a central fingerloop.

Taken together, these structural similarities suggest an evolutionary relationship that will be further discussed in section 1.10.

1.9 - Genetics and RNRs

Ribonucleotide reductases play a central role in the cellular metabolism of every living cell, providing the necessary building blocks for DNA synthesis and repair. Ribonucleotide reduction, however, has to be tightly regulated to ensure a balanced pool of deoxyribonucleotides whenever they are most needed.

We have already described how such regulation is achieved at the protein level, by means of allosteric effectors and complex conformational changes, but a certain level of regulation is also likely to occur at the gene level, allowing cells to sense and quickly respond to changing environmental conditions. Although the biochemical properties of RNRs have been extensively studied, the genetic mechanisms that govern transcription of those genes involved in ribonucleotide reduction are poorly understood. The genetic organization of class I, class II and class III RNRs as well as our current knowledge about its genetic regulation will be discussed in this section.

Note that those genes directly involved in ribonucleotide reduction are commonly annotated *nrd*, which stands for "nucleotide reduction", plus an alphabetical letter assigned according to their chronological characterization.

1.9.1 - Prokaryotic *nrdAB* (Class Ia)

The most extensively studied class Ia RNR genes are, once again, the *nrdAB* genes from *E. coli*. The *nrdA* gene encodes the large α subunit while *nrdB* encodes the smaller β subunit, and they are coordinately expressed as a 3.2 Kb bicistronic mRNA that is transcribed from the *nrdAB* operon (Hanke & Fuchs, 1983a). The –35 and –10 regions of the single promoter found upstream of *nrdA* shows strong similarity to the bacterial consensus promoter sequence (Tuggle & Fuchs, 1986). Transcription from this promoter is coupled to the cell cycle and it is increased by DNA damage and inhibition of DNA replication (either by thymine starvation, addition of hydroxyurea or the use of conditional DNA mutants) (Filpula & Fuchs, 1977; Hanke & Fuchs, 1983b; Tuggle & Fuchs, 1990).

A *cis*-acting AT-rich sequence, located between –139 and –124 relative to the start of transcription and immediately upstream of a 45 bp inverted repeat, was held responsible for coupling *nrdAB* transcription to the cell cycle. DnaA and Fis proteins were also demonstrated to specifically bind to the *nrdAB* promoter region, activating its transcription in a cell cycle-independent manner (Sun *et al.*, 1994).

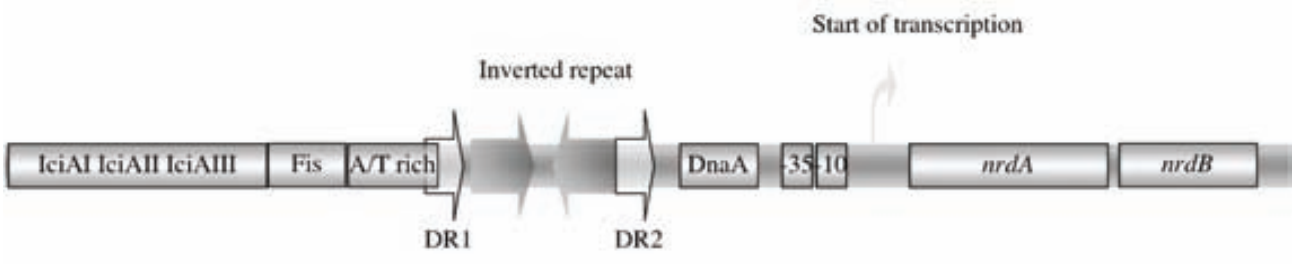

Figure 19. Schematic representation of the *nrdAB* operon from *E. coli*

At first, it was suggested that *nrdAB* was a SOS regulated operon, since its induction patterns greatly resembled those of the SOS induced genes, but up to date, there are no concluding evidences to confirm this suggestion.

Overall, there are at least five upstream cis-acting sites that positively regulate *nrdAB* expression (a Fis binding site, a DnaA binding site, an AT-rich region, an inverted repeat an a 10 bp site), although the transcriptional mechanisms that rule such expression still remain unknown (Jacobson & Fuchs, 1998) (Fig. 19). Later on, the IciA protein from *E. coli* was shown to specifically bind to the *nrdAB* promoter increasing transcription of the *nrdA* and *nrdB* genes (Han *et al.*, 1998).

More recently, the *Streptyomyces coelicolor* class Ia RNR has been shown to be controlled by a genetic riboswitch that represses expression of the *nrdAB* genes in the presence of coenzyme B12 (Borovok *et al.*, 2006).

1.9.2 - Eukaryotic *nrdAB* (Class Ia)

The *nrdA* and *nrdB* genes from the mouse class Ia RNR are located on separate chromosomes and are submitted to separate regulation. The *nrdA* gene is found on chromosome 7 and *nrdB* on chromosome 12. Despite their different regulation, their transcriptional levels increase and decrease in parallel and are cell cycle-dependent. During

G_0/G_1, their mRNA levels are kept at a minimum, but upon entrance into S-phase they are largely increased (Engstrom *et al.*, 1985).

Transcription of R1 is under control of a TATA-less promoter regulated by three different protein complexes. One of them shows S-phase specific binding while the other two are present during the entire cell cycle (Johansson *et al.*, 1995).

Transcription from the R2 promoter is activated at the very beginning of the cell cycle, but only short mRNA transcripts are produced due to a G_1-specific transcriptional block that does not allow mRNA synthesis to progress beyond the first intronic sequence. It is not until the cells reach S-phase that the transcriptional block is overcome and full-length transcripts are produced. There are four DNA-protein interaction regions controlling transcription from the R2 promoter. Three of them are required for the early proliferation specific activation of the promoter, whereas the fourth region has been shown to specifically bind the NF-Y transcriptional factor and it is involved in the S phase-specific release of the block (Filatov & Thelander, 1995).

The promoters of the mouse R1 and R2 genes are also induced in response to DNA damage causing nucleotide excision repair, such as UV irradiation, but not in response to other DNA-damaging agents. Furthermore, UV induction utilizes a mechanism that differs from that of the proliferation specific activation of the R2 promoter (Filatov *et al.*, 1996).

A different picture can be described when analysing enzymatic activity: R1 activity remains constant throughout the cell cycle whereas the enzymatic activity of R2 increases during S-phase and subsequently decreases to very low levels during the remaining phases. The shorter half-live of R2 (3h) compared to that of R1 (>20h) accounts for these differences and, thus, R2 becomes limiting for enzymatic activity (Engstrom *et al.*, 1985). Elimination of the small RNR subunit is achieved by Cdh1-dependent degradation in mitosis (Chabes *et al.*, 2003). More recently, a new mammalian small subunit induced by DNA damage in a p53-dependent manner has been identified (Tanaka *et al.*, 2000). This novel p53R2 is though to provide dNTPs for DNA repair together with R1 (Lin *et al.*, 2004; Nakano *et al.*, 2000).

It is widely accepted that the mechanisms regulating *nrdAB* expression in mouse are also valid for any mammalian RNR, with just some minor exceptions, but they differ from those of the eukaryotic class Ia enzyme found in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* contains two R1 genes (*RNR*1 and *RNR3*) and two R2 genes (*RNR2* and *RNR4*).

The *RNR1* and *RNR2* gene products (designated Y1 and Y2, respectively) are essential for mitotic viability, whereas the *RNR3* gene product (Y3) is not expressed under normal vegetative conditions but it is induced in response to DNA damage and it can suppress a lethal mutation in *RNR1* when overexpressed (Elledge & Davis, 1990).

Surprisingly, the amount of Y1 protein in yeast is coupled to the cell cycle while the levels of Y2 are kept constant, which means that, opposed to what occurred in the mammalian system, it is now the R1 subunit which limits the activity of the holoenzyme (Elledge & Davis, 1989; Elledge & Davis, 1990).

The *RNR4* gene product (Y4) is also quite unusual, it shares 56% sequence identity with Y2 but it lacks 51 amino terminal residues and contains amino acid substitutions in 6 out of 16 conserved residues. In particular, 3 residues essential for iron binding are missing. The purified Y4 protein shows no activity and no tyrosyl radical when assayed together with Y1 or Y3, but it is essential in some genetic backgrounds. It has been shown that Y4 is required to assemble the diiron-Y• cofactor in Y2, and recent studies have suggested that the active form of R2 in *S. cerevisiae* contains a Y2Y4 heterodimer. It is not clear, however, if Y4 is needed to directly deliver iron to Y2 or to direct the proper folding of the heterodimer and stabilize the Y2 structure, allowing binding of iron and formation of the tyrosyl radical (Chabes *et al.*, 2000; Perlstein *et al.*, 2005).

Besides the transcriptional regulation of the yeast *RNR* genes and the allosteric feedback inhibition common to class Ia RNRs, there is another mode to regulate RNR activity in yeast. A novel protein, Sml1, was shown to specifically interact with Y1 in G_1 phase, inhibiting its activity (Chabes *et al.*, 1999). Sml1 is degraded upon entry in S-phase or during DNA damage, which results in de-repression of RNR activity and an increase in the dNTP pools to allow a more efficient repair (Zhao *et al.*, 2001).

1.9.3 - *nrdEF* (Class Ib)

The *nrdEF* genes were first discovered in our laboratory as a second class I ribonucleotide reductase in *S. typhimurium* (Jordan *et al.*, 1994a) and have been found in many prokaryotic organisms since (Torrents *et al.*, 2002). Subsequent characterization of both the *nrdEF* genes and their products led to a reclassification of class I RNRs, which were

subdivided into class Ia (encoded by *nrdAB*) and class Ib (encoded by *nrdEF*) (Jordan *et al.*, 1996b).

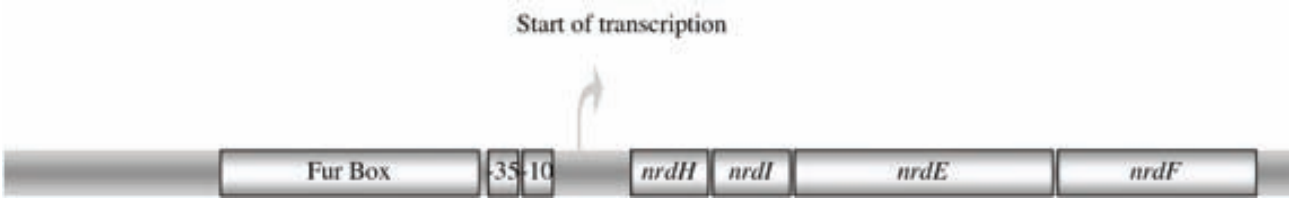

Figure 20. Schematic representation of the *nrdHIEF* operon from *S. typhimurium*

Apart from their particular biochemical properties (discussed above), class Ia and class Ib RNRs can also be differentiated on genetic grounds. Typically, there are four genes involved in class Ib ribonucleotide reduction which, with a few exceptions, arrange as a polycistronic mRNA of about 3.9 Kb. The large α and small β subunits are encoded by the *nrdE* and *nrdF* genes, respectively, whereas *nrdH* encodes an electron donor glutaredoxin-like protein (Jordan *et al.*, 1997) and *nrdI* a protein of yet unknown function (Jordan *et al.*, 1996a) but seemingly essential for class Ib-supported cell growth in *S. typhimurium* (I. Sala, unpublished results). In the *Enterobacteriaceae* family and in most gram-positive bacteria, all four class Ib genes seem to be grouped together in a single locus and they are commonly arranged in a very conserved manner (*nrdHIEF)* (Fig. 20), although some microorganisms with an atypical rearrangement of their class Ib RNR also exist (Torrents *et al.*, 2003).

Initial experiments at our laboratory showed that transcription from the *nrdEF* operon in *S. typhimurium* is silent under normal growth conditions but it is induced in a $nrdA_{ts}$ mutant strain at the restrictive temperature. The induced expression in this mutant, however, is not high enough to suppress the lethal effect of the $nrdA_{ts}$ mutant unless an additional copy of the *nrdEF* operon is present (Jordan *et al.*, 1996a).

Similarly to the *nrdAB* genes, expression of *nrdEF* is induced by hydroxyurea although it is not coupled to DNA replication or DNA damage. The simultaneous presence of two aerobic RNRs (Ia and Ib) among the *Enterobacteriaceae* is yet an unresolved puzzling question. Several experiments from Tao and co-workers have shown an increased expression of the *E. coli nrdEF* genes when growing on minimal medium (Tao *et al.*, 1999). Furthermore, a putative FUR (ferric uptake regulator) binding box has been described

upstream of the *E. coli nrdH* gene (Vassinova & Kozyrev, 2000), and preliminary experiments in our laboratory have shown that expression of the *nrdEF* operon in *S. typhimurium* is enhanced in the presence of iron chelators such as DIP (2,2′-Dipyridyl). FUR is a global transcriptional regulator that uses ferrous iron to repress a variety of genes mainly involved in iron uptake and virulence (Hantke, 2001), thus, a FUR-dependent de-repression of *nrdEF* might occur under those circumstances.

There is also good evidence that the *nrdEF* mRNA levels fluctuate depending on the growth-phase and the composition of the growth medium, that they are enhanced more than 100 fold in *E. coli* strains lacking Trx1 and Grx1 and that they respond to oxidative stress (Monje-Casas *et al.*, 2001). The mechanisms that trigger this increased expression have not been determined yet, but some experiments seem to indicate that the mechanisms up-regulating *nrdEF* expression under oxidative stress do not involve OxyR or SoxR/S, which are key regulators of the adaptive responses to oxidative damage (Storz & Imlay, 1999). These results suggest a potential role for the *Enterobacteriaceae* class Ib enzyme under certain circumstances, such as during the course of an infection, oxidative stress or nutrient starvation, and needs to be further investigated.

In *C. ammoniagenes* (Torrents *et al.*, 2003), transcriptional levels of *nrdHIE* and *nrdF* are also increased 4 to 6 fold in the presence of H_2O_2 and manganese, and two 24 nt sequences resembling the consensus sequence of the transcriptional regulator DtxR (Tao *et al.*, 1994) have been identified upstream of *nrdHIE* and *nrdF*, indicating the presence of a regulatory mechanism responding to metal ions. In a similar manner, experiments carried out in an *nrdD*$^-$ mutant of *Lactococcus lactis* (which also contains the genetic information for a class Ib RNR) have shown that the NrdEF enzyme is active under microaerophilic conditions, suggesting that this enzyme has a very high affinity for oxygen.

On the other hand, class Ib enzymes constitute the sole aerobic RNR of many bacterial organisms where they support their aerobic growth. Examples can be found among species of the genera *Streptococcus*, *Mycoplasma*, *Bacillus*, and *Corynebacterium ammoniagenes*. So far, class Ib RNRs have not been described in any eukaryotic or archaeal organism.

1.9.4 - *nrdJ* (Class II)

All class II RNRs described up to date are encoded by a single gene termed *nrdJ*, with just the exception of the *Pseudomonas aeruginosa* enzyme, which contains a split *nrdJ* gene formed by two adjacent ORFs (*nrdJa* and *nrdJb*) required to constitute the active enzyme (Torrents *et al.*, 2005a). Very little is known about the genetic regulation of class II RNRs, there are just some expression studies performed in *Streptomyces clavuligerus*, *S. coelicolor*, *Mycobacterium tuberculosis* and *P. aeruginosa*. Note that these microorganisms also posses a class I RNR (*P. aeruginosa* contains a class III enzyme as well) and, up to date, there are no experimental evidences from an organism containing only a class II enzyme.

In *S. clavuligerus* and *S. coelicolor*, the transcriptional levels of *nrdJ* are much higher than those of *nrdAB*, and remain constant throughout vegetative growth, whereas transcription of *nrdAB* is low during the early stages of growth and rapidly drops on later stages. In *S. coelicolor*, the class II enzyme has been shown to be the primary RNR class during vegetative growth and to facilitate growth after oxygen limitation, which is consequent with its oxygen-independent nature. Both class Ia and class II RNRs, however, suffice to maintain the cellular viability (Borovok *et al.*, 2002; Borovok *et al.*, 2004).

In *P. aeruginosa*, real-time RT-PCR showed that expression of the class II enzyme is cell-cycle dependent, reaching its maximum during stationary phase, although it is not capable of supporting cell-growth by itself, indicating that growth of *P. aeruginosa* relays mainly on its class I RNR (Torrents *et al.*, 2005a).

Similar results have been obtained in the gram-positive *M. tuberculosis*, where the class Ib enzyme is essential for growth under normal *in vitro* conditions. Class II expression, however, was greatly induced under microaerophilic conditions although initial experiments seem to indicate that it does not play a significant role in virulence and it is not essential for survival under hypoxic conditions *in vitro* (Dawes *et al.*, 2003).

The class II enzymes from *Streptomyces, P. aeruginosa* and *M. tuberculosis* have been suggested to participate mainly in DNA repair, but a much more relevant role is expected in those organisms, such as *Thermotoga maritima*, where a class II enzyme is the only RNR present.

1.9.5 - *nrdDG* (Class III)

The *E. coli nrdDG* genes form a single transcriptional unit of approximately 3 Kb. The *nrdD* gene contains the genetic information for the large α subunit (NrdD) whereas *nrdG* encodes the so-called *activase* (NrdG).

Deletion of the *nrdDG* genes does not impair aerobic growth in *E. coli* but it does under strict anaerobic conditions, indicating the essential role of this enzyme during anaerobic growth (Garriga *et al.*, 1996).

Since class III RNRs are inactivated by oxygen, it is sensible that a regulatory mechanism sensing oxygen concentrations and accordingly activating o repressing *nrdDG* transcription must exist. A recent publication has shown that expression from the *nrdDG* promoter is activated under anaerobic growth, such activation being mediated through the fumarate nitrate reductase (FNR) transcriptional regulator (Boston & Atlung, 2003). Two FNR binding sites upstream of the *nrdD* start codon are proposed although no experimental evidences of FNR binding have been provided yet. Further analysis on the aerobic-anaerobic regulation of the *E. coli* anaerobic RNR will be presented throughout this work.

1.9.6 - *nrdR*. A global transcriptional regulator

While analysing the transcriptional regulation of the *Streptomyces coelicolor* class Ia and class II RNRs (see *nrdJ*), Cohen and co-workers described an additional open reading frame immediately upstream of the *nrdJ* gene that formed a single transcriptional unit together with it. Further analysis demonstrated that deletion of this ORF upregulated transcription of both *nrdJ* and *nrdAB* and, hence, it was termed *nrdR* (Borovok *et al.*, 2004). NrdR was shown to regulate its own expression as well, and it contained an ATP-binding domain very much alike to the allosteric activity site of class Ia RNRs, together with a Zn-ribbon motif. A few months later, Rodionov and Gelfand published an extensive computational analysis of ribonucleotide reductase genes in bacterial genomes, identifying a highly conserved palindromic signal upstream of most *nrd* operons (termed NrdR-box) as well as correlating the presence of this sequence with the occurrence of *nrdR* genes either immediately upstream of *nrd* genes or elsewhere in their genomes (Rodionov & Gelfand,

2005). NrdR-boxes were found upstream of class I, class II and class III RNRs, and the authors suggested that NrdR acts as a global repressor regulating ribonucleotide reductase expression.

In 2006, Grinberg *et al.* (Grinberg *et al.*, 2006) analyzed the NrdR transcriptional regulator from *Streptomyces coelicolor* and showed that NrdR is a nucleotide binding protein which binds up to 0.7 mols of ATP or dATP per mol of NrdR. They also provided experimental evidences that this protein binds to the promoter regions of both *nrdABS* and *nrdRJ* through its zinc finger domain. In view of these results, it is tempting to suggest a novel regulatory mechanism common to all RNRs, where NrdR represses or de-represses *nrd* expression when bound to either dATP/ATP. Back in the late 70's, Fuchs and co-workers already suggested that ribonucleotide reduction was controlled by some compound that accumulated in the cell whenever the rate of DNA synthesis was insufficient to support cell growth (Filpula & Fuchs, 1977), and dATP/ATP might well be such unknown compound.

1.10 - Evolution and RNRs

Up to this point of the introduction you should have a rough idea about the genetic and biochemical properties of ribonucleotide reductases. It is time then to discuss their biological relevance as well as their evolutionary history.

On a preceding RNA world, the advent of an enzyme capable of specifically replacing the 2'-OH group of ribonucleotides with a hydrogen atom had a major impact on life on earth. Such a tiny variation of the ribose chemical properties provided life with a much more stable repository of hereditary information, increasing the fidelity of replication and allowing the generation and maintenance of larger genomes, which, on the long run, resulted in the stepwise development of novel and more complex organisms, i.e.: evolution.

Nevertheless, three different classes of RNRs currently co-exist, each of them utilizing a unique set of co-factors, accessory proteins and oxygen requirements to carry out the very same reaction. Is then the modern scenario the result of convergent evolution acting on three independently evolving enzymes or, on the other hand, ribonucleotide reductases evolved from a single ancestral enzyme to originate the existing RNR classes through divergent evolution?

Despite the lack of similarity at their primary sequences, class I, II and III RNRs have in common the use of a thiyl radical and reducing cysteines at their active site, a very tight substrate specificity regulation, a very similar mechanism of reaction as well as a highly conserved overall three-dimensional structure. It could be argued that these similarities simply reflect the mechanistic requirements for ribonucleotide reduction and are due to convergent evolution, but it is widely accepted that the conserved position of the active site cysteines, the striking similarities regarding the allosteric regulation pathways as well as the use of a conserved chemistry suffice to support their evolutionary relatedness (Reichard, 2002; Stubbe *et al.*, 2001). The mechanism of radical initiation, on the other hand, is not conserved among the three RNR classes and it is probably the result of different adaptive strategies to the environment.

Furthermore, the crystal structure of the *L. leichmannii* class II RNR (Sintchak *et al.*, 2002), the last to be solved, confirms the general view that the common evolutionary origin of RNRs is reflected at their tertiary structure level. In fact, all three classes share the unique ten-stranded β/α barrel containing the active site with its characteristic finger loop (Fig. 15).

If we assume that RNRs evolved by divergent evolution, which class most closely resembles the ancestral "ur"-reductase?

The evolutionary origin of ribonucleotide reductases is still an open debate, but there is general agreement about the nature of the driving force influencing the divergence of RNRs: **oxygen**. Under the assumption that DNA appeared on our planet before the advent of oxygen, the first reductase had to be an anaerobic enzyme. Class III RNRs are strictly anaerobic and class II enzymes can function either with or without oxygen, therefore, both classes are likely candidates to be the ancestral reductase. Class I RNRs, which are oxygen-dependent, are excluded since they probably arose only after the emergence of photosynthesis created an O_2 atmosphere.

Peter Reichard has strongly argued in favour of class III RNR being the closest relative to the ur-reductase mainly on the basis of its biochemical requirements and the earth early atmospheric conditions (Reichard, 1993; Reichard, 1997; Reichard, 2002): (i) The anaerobic class III enzyme utilizes S-adenosylmethionine (SAM) to initiate the reaction, a structurally more simple compound than S-adenosylcobalamin (AdoCbl), whose biosynthesis involves more steps and enzymes; (ii) Class III RNRs depend on a Fe-S cluster to generate the

glycyl radical, and sulfur and iron are known to be predominant in earth's early atmosphere, whereas cobalt, used by class II RNRs, was rare; (iii) Formate, one of the simplest organic reductants, is the ultimate hydrogen donor of class III RNRs, which do not require any accessory proteins such as the thioredoxin and glutaredoxin systems employed by class I and II RNRs; (iv) The allosteric regulation is much less specialized in class III enzymes, where each allosteric site regulates both specificity and activity. In class I and II RNRs, however, one single site regulates substrate specificity and the other, when present, is in charge of regulating the overall activity of the enzyme; and (v) Class III RNR is structurally related to PFL, which is a very ancient enzyme involved in anaerobic metabolism. Reichard suggested that the anaerobic RNR could have evolved from a primitive form of PFL.

On the other hand, the lesser structural complexity of the monomeric class II enzyme from *L. leichmannii* and the fact that AdoCbl, despite its higher complexity and more demanding synthesis, generates a 5'-deoxyadenosyl radical more readily than SAM without the aid of any accessory protein, argues in favour of class II being the ancestral stage, although the close similarities between the structures of class I and II RNRs speak against it, since class I enzymes are thought to be an evolutionary latecomer. It is worth noticing that the arguments proposed by Reichard are mere speculations, and since no experimental or statistical data has been provided so far, they can be easily criticized by those in favour of class II RNRs (Poole *et al.*, 2002).

More recently, Torrents and co-workers analysed the evolutionary history of RNRs by comparing their three dimensional structures and inferring their phylogenetic relationships (Torrents *et al.*, 2002). Although not yet conclusive, the results obtained by Torrents and co-workers support divergent evolution of RNRs from a common ancestor together with a great deal of lateral transfer, and suggest that a class III enzyme is the most likely candidate to represent the ur-reductase. Since both class II and class III enzymes are found among bacteria and archaea, they suggest that both classes co-existed in the Last Universal Common Ancestor (LUCA) and that duplication of the ancestral ur-reductase to generate class II RNR must have occurred at a very early stage (Fig. 21).

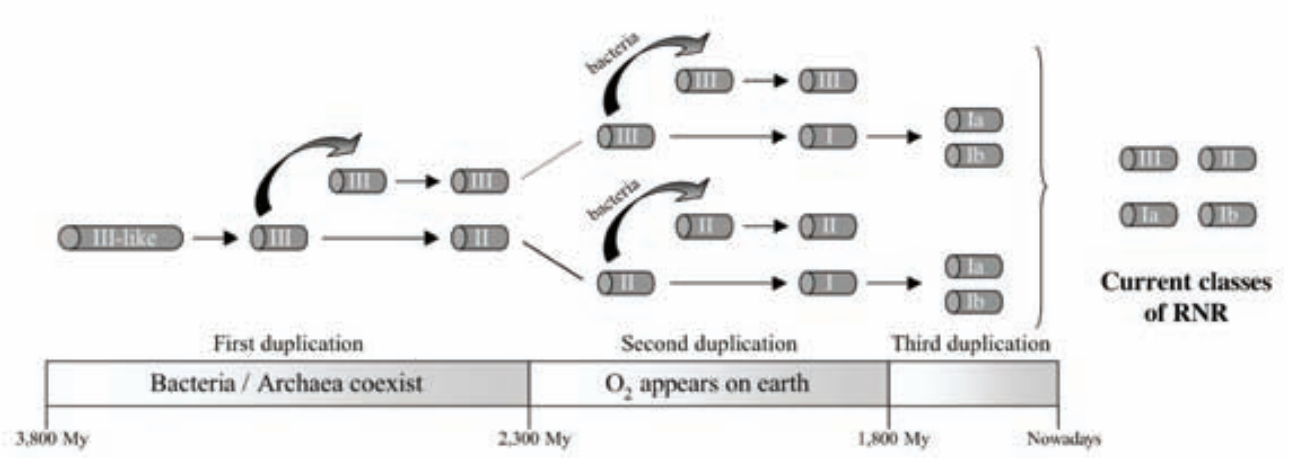

Figure 21. Origin by duplication of the four known *nrd* gene classes under the assumption that they derived from an unknown primitive class III-like gene. Origin of class I RNR is depicted as a duplication event either from a class III or a class II enzyme. Further duplication of a class I gene gave rise to classes Ia and Ib

The advent of class II may have allowed the colonization of the first aerobic oasis created by the photolysis of water. Later on, and coinciding with the transition towards an aerobic atmosphere, a second duplication step, most likely from a class II RNR, resulted in a class I enzyme, which provided a selective advantage during aerobic growth since it relieved organisms from the costly synthesis of AdoCbl. According to Torrents *et al*., fusion of a bacterium carrying a class Ia RNR with an archaebacterium would have produced the first eukaryotic cell which contained all three RNR classes, although class II and class III subsequently degenerated, since all eukaryote RNRs belong to class Ia (with the sole exception of *Euglena gracilis*, which contains a class II RNR (Torrents *et al.*, 2006)). It is also likely that further duplication of a bacterial class Ia RNR originated the prokaryotic class Ib enzyme.

Table II depicts the occurrence of ribonucleotide reductases throughout the three domains of life (see also: http://rnrdb.molbio.su.se). As mentioned above, archaeal species contain either class II or class III RNRs (or both in a vast majority of them), but not class I (the class I RNR of *Halobacterium* seems to be the result of a lateral transfer event) (Torrents *et al.*, 2002). Class Ia, Ib, II and III RNRs are heterogeneously spread among bacteria, where

more than one RNR class can be simultaneously housed by a single organism. In this respect, every possible pairwise combination of RNRs is found among the members of bacteria.

Moreover, the genomes of several microorganisms (*Pseudomonas aeruginosa*, *Clostridium acetobutylicum* or *Clostridium difficile* among them) have been shown to contain all three RNRs (Jordan *et al.*, 1999). The presence of a specific RNR class in a given bacterium reflects its ability to grow either on the presence or the absence of oxygen (or both), although the specific combination found in aerobe (I or II or both), anaerobe (II or III or both) and facultative microorganims (I+II or I+III or II+III) does not follow any apparent pattern. Eukarya exhibits the least diversity since class Ia is the only RNR class found among its members (*Euglena gracilis* being the only exception). Finally, the RNR classes from viruses reflect those of their hosts.

The simultaneous occurrence of different RNR classes within a single genome and, particularly, in those species bearing combinations of RNR classes that can be used under the same environmental conditions (i.e.: I+II, II+III, Ia+Ib, and I+II+III) poses the question of whether these extra enzymes provide a selective advantage in certain growth conditions or are just mere evolutionary relics.

Table II. Sample distribution of RNRs across the three domains of life. Ocurrence of ribonucleotide reductases are from http://rnrdb.molbio.su.se/, last updated 2007-02-27. (Adapted from Torrents et al., 2002)

Species	Metabolism	Sequence status	RNR class	accession number
ARCHAEBACTERIA				
Crenarchaeota				
Aeropyrum pernix	AE	G	II	NC_000854
Euryarcheota				
Archaeoglobus fulgidus	AN	G	II	NC_000917
Halobacterium sp.	AE	G	Ia, II	NC_002607
Methanothermobacter thermautotrophicus	AN	G	II, III	NC_000916
Methanococcus maripaludis	AN	G	III	NC_005791
Pyrococcus abyssi	AN	G	II, III	NC_000868
Pyrococcus furiosus	AN	G	II, III	NC_003413
Pyrococcus horikoshii	AN	G	II, III	NC_000961
EUBACTERIA				
Actinobacteria				
Bifidobacterium longum	AN	G	Ib, III	NC_004307
Corynebacterium ammoniagenes	AE	S	Ib	Y09572
Corynebacterium diphtheriae	AE	G	Ib(2E)	NC_002935
Corynebacterium efficiens	AE	G	Ib, III	NC_004369
Mycobacterium avium	AE	G	Ib, II	NC_008595
Mycobacterium bovis	AE	G	Ib, II	NC_008769
Mycobacterium leprae	AE	G	Ib, II	NC_002677
Mycobacterium smegmatis	AE	G	Ib, II	NC_008596
Mycobacterium tuberculosis	AE	G	Ib(2F), II	NC_000962
Propionibacterium acnes	AN	G	Ia, II, III	NC_006085
Streptomyces clavuligerus	AE	S	II	AJ224870
Streptomyces coelicor	AE	G	II	NC_003888
Aquificales				
Aquifex aeolicus	AE	G	Ia	NC_000918
Cyanobacteria				
Anabaena variabilis	AE	G	II	NC_007413
Nostoc sp.	AE	G	II	NC_003272
Prochlorococcus marinus	AE	G	II	NC_007577
Synechocystis sp.	AE	G	Ia	NC_000911
Bacteroidetes/Chlorobi group				
Bacteroides fragilis	AN	G	Ia, II, III	NC_003228
Chlorobium chlorochromatii	AN	G	II	NC_007514
Chlorobium tepidum	AN	G	II	NC_002932
Porphyromonas gingivalis	AN	G	II, III	NC_002950
Salinibacter ruber	AE	G	Ia, II	NC_007677
Firmicutes (gram-positive bacteria)				
Bacillus anthracis	AE	G	Ib, III	NC_005945
Bacillus cereus	FA	G	Ib, III	NC_003909
Bacillus halodurans	AE	G	Ia, II	NC_002570

Species	Metabolism	Sequence status	RNR class	accession number
Bacillus subtilis	AE	G	Ib(2×)	NC_000964
Clostridium acetobutylicum	AN	G	Ia, II, III	NC_003030
Clostridium difficile	AN	g	Ia, II, III	NZ_AAML0
Clostridium perfringens	AN	G	Ia, III(2x)	NC_003366
Clostridium tetani	AN	G	II, III	NC_004557
Enterococcus faecalis	FA	G	Ib, III	NC_004668
Lactobacillus leichmannii	FA	S	II	L20047
Lactobacillus plantarum	FA	G	Ib, III	NC_004567
Lactococcus lactis	FA	G	Ib, III	NC_002662
Listeria monocytogenes	FA	G	Ia, III	NC_003210
Mycoplasma gallisepticum	FA	G	Ib	NC_004829
Mycoplasma genitalium	FA	G	Ib	NC_000908
Mycoplasma hyopneumoniae	FA	G	Ib	NC_006360
Mycoplasma penetrans	FA	G	Ib, III	NC_004432
Mycoplasma pneumoniae	FA	G	Ib	U00089
Mycoplasma pulmonis	FA	G	Ib	NC_002771
Staphylococcus aureus	FA	G	Ib, III	NC_002745
Staphylococcus epidermidis	FA	G	Ib, III	NC_004461
Streptococcus agalactiae	FA	G	Ib(x2), III	NC_004116
Streptococcus mutans	FA	G	Ib, III	NC_004350
Streptococcus pneumoniae	FA	G	Ib, III	NC_003098
Streptococcus pyogenes	FA	G	Ib(2×), III	NC_002737
Thermoanaerobacter tengcongensis	AN	G	II, III	NC_003869
Plantomyces/Chlamydia group				
Chlamydia trachomatis	AE	G	Ia	NC_000117
Chlamydophila pneumoniae	AE	G	Ia	NC_002491
Proteobacteria				
Alpha subdivision				
Agrobacterium tumefaciens	AE	G	Ib, II	NC_003304
Bradyrhizobium japonicum	AE	G	Ia, II	NC_004463
Brucella abortus	AE	G	Ib, II	NC_006932, NC_006933
Caulobacter crescentus	AE	G	Ia, II	NC_002696
Erythrobacter litoralis	AE	G	Ia	NC_007722
Rhodobacter sphaeroides	FA	g	II	NZ_AAMF0
Rhodopseudomonas palustris	FA	G	Ia, II, III	NC_008435
Rhodospirillum rubrum	FA	G	Ia, II, III	NC_007643
Rickettsia prowasekii	AE	G	Ia	C71655
Beta subdivision				
Bordetella pertussis	AE	G	Ia	NC_002929
Burkholderia thailandensis	AE	G	Ia, II, III	NC_007650, NC_007651
Chromobacterium violaceum	FA	G	Ia, II, III	NC_005085
Neisseria gonorrhoeae	AE	G	Ia	NC_002946
Neisseria meningitidis	AE	G	Ia	NC_003116
Ralstonia eutropha	FA	G	Ia, II, III	NC_007347, NC_007348
Rhodoferax ferrireducens	FA	G	Ia, II, III	NC_007908
Thiobacillus denitrificans	FA	G	Ia, II, III	NC_007404
Delta & Epsilon subdivision				
Bdellovibrio bacteriovorus	AE	G	Ia, II, III	NC_005363

Species	Metabolism	Sequence status	RNR class	accession number
Campylobacter jejuni	AE	G	Ia	NC_003912
Desulfovibrio desulfuricans	AN	G	II, III(2D)	NC_007519
Geobacter sulfurreducens	AN	G	II	NC_002939
Helicobacter pylori	AE	G	Ia	P55982
Gamma subdivision				
Actinobacillus pleuropneumoniae	FA	g	2xIa, III	NZ_AACK0
Chromohalobacter salexigens	AE	G	Ia, Ib	NC_007963
Erwinia carotovora	FA	G	Ia, Ib, III	NC_004547
Escherichia coli (Enterobacteriacea)	FA	G	Ia, Ib, III	NC_000913
Francisella tularensis	AE	G	Ia	NC_006570
Haemophilus influenzae	FA	G	Ia, III	P43754, A64047
Legionella pneumophila	AE	G	Ia	NC_006368
Pasteurella multocida	FA	G	Ia, III	NC_002663
Photobacterium profundum		G	Ia, II, III	NC_006370, NC_006371
Photorhabdus luminescens		G	Ia, Ib, III	NC_005126
Pseudomonas aeruginosa	FA	G	Ia, II, III	NC_002516
Pseudomonas putida	FA	G	Ia	NC_002947
Salmonella typhimurium	FA	G	Ia, Ib, III	NC_003197
Shewanella denitrificans	FA	G	Ia, III	NC_007954
Shigella dysenteriae	FA	G	Ia, III	NC_007606
Shigella flexneri	FA	G	Ia, Ib, III	NC_004337
Shigella sonnei	FA	G	Ia, Ib, III	NC_007384
Vibrio cholerae	FA	g	Ia, III	NZ_AAUT0
Xylella fastidiosa	AE	G	Ia	NC_004556
Yersinia pestis	FA	G	Ia, Ib, III	NC_003143
Spirochaetes				
Borrelia burgorferi	AE	G	Ia	NC_001318
Treponema pallidum	AE	G	Ia	NC_000919
Thermotogales				
Thermotoga maritima	AN	G	II	Y12877
Thermus/Deinococcus group				
Deinococcus radiodurans	AE	G	Ib, II	AE001826, D75281
Thermus thermophilus	FA	G	Ia, II	NC_006461
Eubacteria viruses				
Mycobacteriophage L5			II	S30995
Phage T4		G	Ia, III	AF158101
Roseophage S101			II	NC002519
EUKARYOTES and EUKARYOTA VIRUSES				
All			Ia	
Euglena gracilis		S	II	CAF05662
Dictyostelium discoideum		G	Ia, II	NC_007087 to NC_007092

Notes: AE: aerobe; AN: anaerobe; FA: facultative anaerobe; G/g: genome sequence available (complete/partial); S: gene sequence known.

1.11 - Ribonucleotide Reductases as Biomedical Targets

As stated before, ribonucleotide reductases are essential enzymes in all living organisms. They are needed for deoxyribonucleotide synthesis during the eukaryotic S-phase and prokaryotic DNA replication, as well as for DNA repair after DNA damage. Such essentiality makes them ideal targets for drugs designed to inhibit cell growth, either in abnormal proliferative processes (cell cancer) or during an infection caused by biological agents such as bacteria, viruses or protozoic organisms. A better understanding of the molecular mechanisms regulating the expression and activity of these enzymes may, therefore, settle the basis towards a new generation of anti-proliferative drugs.

Nowadays, several ribonucleotide inhibitors are already available or under study, and they can be grouped according to their specific modes of action. For a more extensive review, see (Cerqueira *et al.*, 2005; Eklund *et al.*, 2001).

R2-specific catalytic inhibitors

This group of inhibitors specifically interacts with the R2 subunit and prevents radical formation either by scavenging the tyrosyl radical in R2 (one electron reductants), or by chelating the iron from the binuclear centre, which leads to accumulation of Apo R2. Hydroxyurea, hydroxylamine, hydroquinones and nitric oxide are examples of radical scavengers, while deferrioxamine or triapine constitute examples of potentially useful iron chelators.

R1-specific catalytic inhibitors

This group of inhibitors specifically interacts with the R1 subunit and impairs its catalytic activity by means of three different mechanisms.

(i) Some inhibitors within this category are inactivators of sulfhydryl groups. These inhibitors are small molecules that covalently bind to the active site cysteines, irreversibly inactivating the R1 subunit. Caracemide and cisplatin are two of the most popular sulfhydryl inactivators.

(ii) Another group of R1-specific inactivators is that of ATP analogues that interact with the allosteric sites of RNRs, inhibiting the activity of the enzyme. Fludarabine and clofarabine, among others, are examples of this type of inhibitors.

(iii) The third group comprises substrate analogues, or suicide inhibitors, which are recognized at the active site of the enzyme and react with it, originating harmful side-reactions that inactivate the enzyme. This type of inhibitors has proven very potent mechanism-based inhibitors. Gemcitabine is a nucleoside analogue which has been approved for the treatment of pancreatic and lung cancer.

Antisense inhibitors

These inhibitors are basically antisense oligonucleotides that bind to either R1 or R2 mRNA and prevent expression of the coded protein or even lead to its destruction. Since antisense technology is relatively recent, this type of inhibitors is still under development, although very promising projects are under way.

Dimerization inhibitors

This group of inhibitors takes advantage of previous RNR investigations where the carboxyl end of R2 was shown to be involved in formation of the R1-R2 complex (Uhlin & Eklund, 1994). These are short peptidomimetic drugs designed to compete with R2 during formation of the R1-R2 complex.

Most of the studies concerning RNR inhibition are centered towards tumor suppression and inhibition of class Ia RNRs, but as more bacterial and viral genomes are being published and occurrence of RNR classes other than Ia in these organisms becomes more apparent, the development of class-specific RNR inhibitors may allow the treatment of bacterial or parasitic infections without interfering with the host's enzyme.

2. - Objectives

The concurrence of apparently redundant RNRs within a single genome as well as the regulatory mechanisms that drive their differential expression are two of the most intriguing questions left for those studying ribonucleotide reduction. In this work we have attempted to provide some answers to these questions by studying class Ib RNR redundancy in the Gram-positive bacterium *Streptococcus pyogenes* (Part I), and class III RNR anaerobic activation in the enteric bacterium *Escherichia coli* (Part II).

Specific objectives for each part are:

Part I - Class Ib ribonucleotide reductase redundancy in *S. pyogenes*

- ✓ Assess the operonic nature and transcriptional expression of the class Ib *nrd* genes from *S. pyogenes*
- ✓ Analyze the enzymatic activity and biochemical properties of each class Ib RNR system from *S. pyogenes*
- ✓ Determine the *in vivo* essentiality of the class Ib *nrd* genes from *S. pyogenes*
- ✓ Provide a plausible explanation for class Ib RNR redundancy in *S. pyogenes*

Part II - Anaerobic activation of the *E. coli* class III ribonucleotide reductase

- ✓ Analyze the differential expression of the *E. coli* class III RNR *nrd* genes under aerobic and anaerobic growth
- ✓ Identify the regulatory mechanisms modulating the expression of the anaerobic class III RNR *nrd* genes from *E. coli*
- ✓ Elucidate the mechanism by which such regulation takes place

3. - Part I - *Streptococcus pyogenes* Class Ib RNR

3.1 - Brief Introduction

Streptococcus pyogenes (Group A Streptococcus [GAS]) is a Gram-positive bacterium with low GC% content belonging to the *Streptococcaceae* family. It is a human pathogen responsible for many diverse infections, causing mild disease (pharyngitis and impetigo), severe life-threatening infections (toxic shock-like syndrome) and post-infection sequelae (rheumatic fever and glomerulonephritis). It is a facultative anaerobe, capable of growing in both aerobic an anaerobic environments, and its genome (Ferretti *et al.*, 2001) harbours three different clusters of genes involved in ribonucleotide reduction. The first cluster contains the genes for the anaerobic enzyme (*nrdDG*) but, surprisingly, each of the two other clusters contains the genes to form a class Ib RNR (*nrdFIE** and *nrdHEF*). A second and isolated copy of an *nrdI* gene (*nrdI2* in this work) has also been annotated elsewhere in the chromosome. It is very common to find concurrence of different RNR classes within a single microorganism (Torrents *et al.*, 2002) but in just a very few cases a duplication of *nrd* genes belonging to the same class has been described (Wang *et al.*, 1997; Yang *et al.*, 1997).

Streptococcus pyogenes is the first prokaryotic organism where two distinct and complete RNRs of the same class are studied. A second set of genes encoding almost the same enzyme might be of great importance under different environmental conditions or might be simply redundant, but there are two more features that make this microorganism unique. First, *S. pyogenes* not only has a duplication of genes but it also arranges them in a way that deviates from the common *nrdHIEF* structure and, second, three residues involved in iron binding are not conserved within the small subunit of the *nrdFIE** cluster. Although very little is known about the genetics of *S. pyogenes* and just a few tools are available for its manipulation, in this study we have attempted to analyze both the *in vivo* and *in vitro* functionality of the *S. pyogenes* class Ib ribonucleotide reductases.

3.2 - Results

3.2.1 - Sequence analysis

After the publication in 2001 of the *Streptococcus pyogenes Rosenbach* M1 complete genome (Ferretti *et al.*, 2001), seven genes putatively encoding for class Ib ribonucleotide reductase related proteins where identified in three different regions of the genome (Fig. 22).

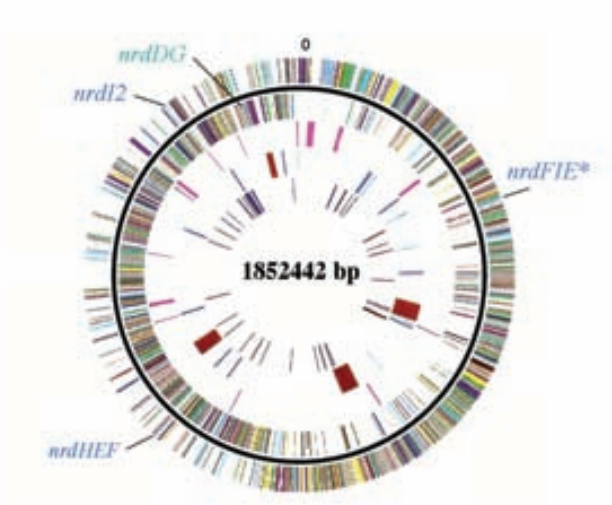

Figure 22. Circular representation of the *S. pyogenes* strain SF370 genome. *Outer circle*, predicted coding regions transcribed on the forward (clockwise) DNA strand. *Second circle*, predicted coding regions transcribed on the reverse (counterclockwise) DNA strand. *Third circle*, stable RNA molecules. *Fourth circle*, mobile genetic elements: burgundy, bacteriophage; blue, transposons/IS elements; light cyan, transposons/IS elements (pseudogenes). *Fifth circle*, known and putative virulence factors: purple, previously identified ORFs; brown, ORFs identified as a result of genome sequence. The lines in each concentric circle indicate the position of the represented feature. (Adapted from Ferretti *et al.*, 2001)

The first region spanned over 4.5 kbp and comprised the *nrdHEF* cluster. The *nrdH* gene encoded a putative glutaredoxin-like protein of 73 aa and 8.2 kDa. The *nrdE* gene encoded the large catalytic subunit of the holoenzyme, with 720 aa and a predicted molecular weight of 81 kDa. It had all the essential cysteines in the active site of the enzyme as well as the conserved C-terminal tyrosines, required for the transfer of the radical from the small subunit, and the residues that form the allosteric specificity site (Table III). The small subunit of the enzyme, encoded by the *nrdF* gene, is a protein of 319 aa with a expected molecular

weight of 37 kDa bearing all the conserved residues for iron binding, radical generation and radical transfer (Table IV).

The second region comprised the *nrdFIE** cluster (denoted here as *nrdF**, *nrdI** and *nrdE**) and spanned over 4 kbp. The *nrdF** gene encoded a ribonucleotide reductase class Ib small subunit with a predicted molecular weight of 39 kDa and 337 aa. Surprisingly, not all-important residues for the proper function of the enzyme are conserved. NrdF* turned out to have an aminoacid-substitution in three out of the six important residues involved in iron binding as well as in one of the residues involved in radical stability (Table IV); instead of Glu115 it had Val116, instead of Glu204 it had Pro176, instead of Glu238 it had Lys210, and instead of Phe208 it had Leu180. All other residues for radical stability and transfer seemed, however, to be highly conserved. The *nrdI** gene encoded an unknown protein of 18 kDa and 162 aa which is 75 % similar to that of *E. coli*. Finally, the *nrdE** gene also encoded a ribonucleotide reductase large subunit with all active site residues highly conserved (Table III). It was a protein of 727 aa and a predicted molecular weight of 81 kDa.

A second copy of an *nrdI* gene (*nrdI2*) was also annotated elsewhere in the genome (Ferretti *et al.*, 2001). This copy was found isolated from any other RNR cluster in the *S. pyogenes* chromosome and encoded a protein of 160 aa and 17.7 kDa (Fig. 22).

Table III. Important residues in R1.

E. coli NrdA	*S. typhimurium* NrdE	*S. pyogenes* NrdE	*S. pyogenes* NrdE*	*S. pneumoniae* NrdE	Function
S224	S168	S171	S169	S171	Active site
C225	C169	C172	C170	C172	Active site, redox active cysteine
D232	D176	D179	D177	D179	Allosteric specificity site
L234	M178	M181	M179	M181	Allosteric specificity site
I237	I181	I184	I182	I184	Allosteric specificity site
K246	Q190	Q193	Q191	Q193	Allosteric specificity site
R262	R206	R209	R207	R209	Allosteric specificity site
I268	I212	I215	I213	I215	Allosteric specificity site
R269	K213	K216	K214	K216	Allosteric specificity site
T276	S220	S223	T221	S233	Allosteric specificity site
R329	R271	R276	R274	R276	R2 interaction area
N437	N377	N380	N380	N380	Active site
L438	L378	L381	L381	L381	Active site
C439	C379	C382	C382	C382	Active site, transient thiyl radical
E441	E381	E384	E384	E384	Active site, base in the catalysis
C462	C406	C409	C409	C409	Active site, redox active cysteine
L464	L408	L411	L411	L411	Active site
P621	P579	P580	P582	P580	Active site
Y730	Y683	Y697	Y687	Y697	Electron transfer chain
Y731	Y684	Y698	Y688	Y698	Electron transfer chain
C754	C700	C714	C721	C714	Redox active cysteine, Trx/Grx interaction
C759	C703	C717	C724	C717	Redox active cysteine, Trx/Grx interaction

Table IV. Important residues in R2.

E. coli **NrdB**	*S. typhimurium* **NrdF**	*S. pyogenes* **NrdF**	*S. pyogenes* **NrdF***	*S. pneumoniae* **NrdF**	**Function**
W48	W31	W30	W49	W30	Electron transfer chain
T81	T64	T63	T82	T63	Hydrophobic pocket
L82	L65	L64	L83	L64	Hydrophobic pocket
L83	L66	L65	L84	L65	Hydrophobic pocket
D84	D67	D66	D85	D66	Iron ligand, electron transfer chain
S85	T68	T67	T86	T67	Hydrophobic pocket
Q87	Q70	Q69	Q88	Q69	Hydrophobic pocket
E115	E98	E97	**V116**	E97	**Iron ligand**
H118	H101	H100	H119	H100	Iron ligand, electron transfer chain
Y122	Y105	Y104	Y123	Y104	Stable tyrosyl radical
E204	E158	E157	**P176**	E157	**Iron ligand**
I206	F160	F159	F178	F159	Hydrophobic pocket
R207	L161	L160	L179	L160	Hydrophobic pocket
F208	F162	F161	**L180**	F161	**Hydrophobic pocket, radical stability**
Y209	Y163	Y162	Y181	Y162	Hydrophobic pocket
S211	G165	G164	G183	G164	Hydrophobic pocket
F212	F166	F165	F184	F165	Hydrophobic pocket
L233	L187	L186	L205	L186	Hydrophobic pocket, R1 interaction
I234	I188	I187	I206	I187	Hydrophobic pocket
A235	I189	I188	L207	I188	Hydrophobic pocket
R236	R190	R189	R208	R189	Hydrophobic pocket, R1 interaction
D237	D191	D190	D209	D190	Electron transfer chain
E238	E192	E191	**K210**	E191	**Iron ligand**
H241	H195	H194	H213	H194	Iron ligand
Q345	N293	N291	N311	N291	R1 interaction
V346	H294	H292	H312	H292	R1 interaction
A347	D295	D293	D313	D293	R1 interaction
P348	F296	F294	F314	F294	R1 interaction
Q349	F297	F295	F315	F295	R1 interaction
E350	S298	S296	S316	S296	R1 interaction
Y356	Y304	Y302	Y322	Y302	Electron transfer chain

3.2.2 - Transcriptional analysis

Our next step was to demonstrate that all class Ib RNR regions were simultaneously transcribed and to assess the operonic organization of each cluster. Total RNA from strain SF370 was extracted at early exponential phase and used to perform non-quantitative RT-PCR experiments.

Primer pairs were selected to bracket the intergenic junctions between *nrdHE*, *nrdEF*, and *nrdFIE**. Any PCR fragment generated by these oligonucleotides would therefore be derived from a polycistronic transcript (Fig. 23A).

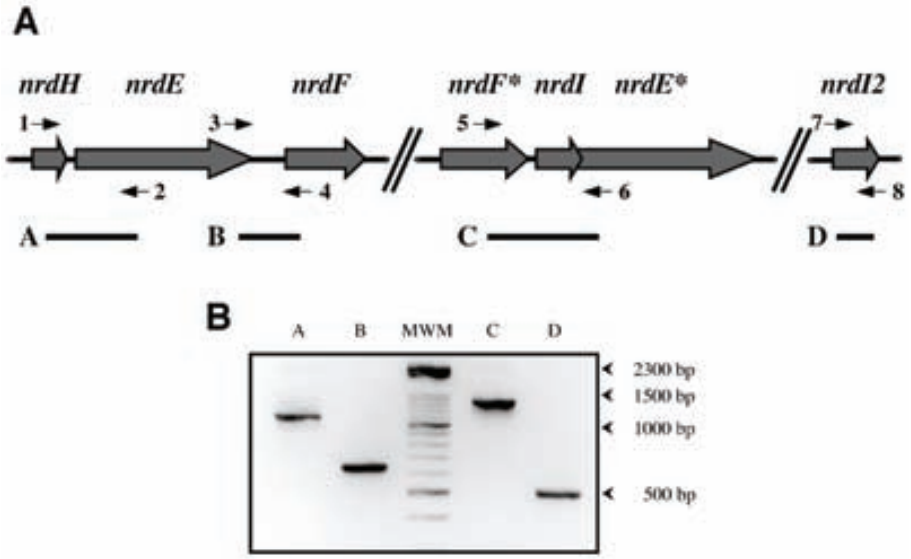

Figure 23. A. Schematic organization of the *S. pyogenes* class Ib *nrd* genes. Black arrows indicate the position and number of the primers used for RT-PCR amplification and A, B, C and D black lines show the position and length of the expected transcripts. **B.** Linkage RT-PCR of the *nrdHE* (lane A), *nrdEF* (lane B), and *nrdFIE* (lane C) junctions and the *nrdI2* gene (lane D). The linkage strategy is outlined in **A**.

An amplicon spanning the *nrdHE* junction was detected by using primer nº 2 to generate the cDNA and primers nº 1 and nº 2 (Table XI) for the PCR reaction (Table XI and Fig. 23B, lane A). Similarly, a PCR product bracketing the junction between *nrdE* and *nrdF* could also be detected with primers nº 3 and nº 4 (Table XI and Fig. 23B, lane B).

Using primer nº 6 to generate cDNA templates reversed transcribed and the primer pair 5-6 (Table XI) to amplify that template, we were also able to detect amplicons spanning the *nrdFIE** intergenic junction (Fig. 23B lane C).

An RT-PCR reaction for the second *nrdI2* gene was also carried out with primers nº 7 and nº 8 (Table XI), and the corresponding transcript could also be detected (Fig. 23B, lane D). The *nrdI2* product was sequenced to ensure its correct amplification.

A series of control experiments conducted concurrently with each set of RT-PCR assays confirmed the quality of the samples and guaranteed the specificity of each component of the RT-PCR reaction. (i) When DNAse I-treated total RNA served as a template, no PCR products were detected, ensuring that residual genomic DNA had not contaminated the starting RNA preparations (not shown); (ii) the expected amplicons were detected when genomic DNA acted as the template, demonstrating the fidelity of the PCR primers (data not shown); and (iii), the inability to amplify the desired product when RT was omitted validated the compulsory requirement of this enzyme for the initiation of cDNA synthesis.

These results indicated the presence of a polycistronic transcript for each cluster of genes as well as their simultaneous transcription under standard laboratory growth conditions.

3.2.3 - Construction of *S. pyogenes nrd* mutants

A set of suicide vectors containing internal fragments of the *S. pyogenes nrdE, nrdF, nrdF*, nrdI*, nrdE** and *nrdI2* genes were constructed and introduced into the *S. pyogenes* strain SF370 (see experimental procedures) in an attempt to obtain integrational mutants for each of the above mentioned *nrd* genes. However, after 48 hours of incubation on antibiotic-containing agar plates, no transformants could be detected. In a similar way, selection of *covR* mutants (our positive control gene) by the same procedure was quite unsatisfactory since colonies were recovered at frequencies as low as $1x10^{-8}$.

3.2.4 - Radical and iron content of NrdF and NrdF*

Once demonstrated that all class Ib *nrd* genes in *S. pyogenes* were actively transcribed, we decided to check whether they were capable of forming an active holoenzyme. The *nrdE* and *nrdF* genes from both the *nrdHEF* and *nrdFIE** operons were cloned,

overproduced and purified as described in the experimental procedures. After different purifications steps, all purified proteins showed the expected theoretical molecular weight. The purity of the samples in all cases was over 90% (Fig. 24). Attempts to overproduce and purify the NrdI* or the NrdI2 proteins were unsuccessful since the overproduced proteins were continuously recovered in the insoluble fraction of the bacterial extract, regardless of the incubation temperature or the IPTG concentrations used (not shown).

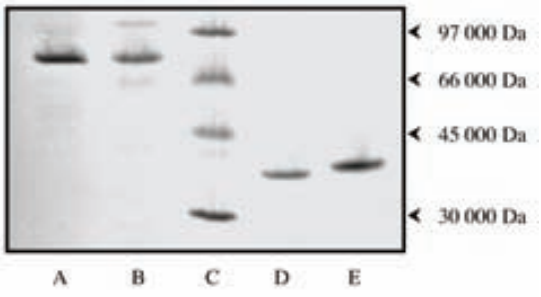

Figure 24. SDS-PAGE of the purified NrdE (A), NrdE* (B), NrdF (D) and NrdF* (E) proteins. Lane C, marker protein (Amersham Pharmacia Biotech LMW marker kit; 30,000, 45,000, 66,000 and 97,000 Da)

Purified NrdF protein showed a light absorption spectrum typical of a diiron-tyrosyl radical RNR. Charge-transfer bands of the μ-oxobridged diferric center were observed at 325 and 370 nm, and weaker bands at 500 and 600 nm (Fig. 25A). The tyrosyl radical showed a sharp peak at 407 nm. We also registered the EPR spectrum of the NrdF protein (Fig. 26), and the tyrosyl radical proved to be a typical class Ib radical similar to those found in *S. typhimurium, C. ammoniagenes* and *M. tuberculosis* (Huque *et al.*, 2000; Jordan *et al.*, 1994b; Liu *et al.*, 1998). At 95 K, the radical saturated at one order of magnitude less power than does the *E. coli* NrdB at 95 K (4.7 mW compared to 54.0 mW, data not shown) but similar to the *S. typhimurium* and *C. ammoniagenes* NrdF proteins. The *S. pyogenes* NrdF radical signal, like other class Ib RNRs, retained the hyperfine structure at 95 K as well resolved as at 10-20 K. Analysis of the NrdF iron content showed approximately 1.1 Fe/monomer (Table V).

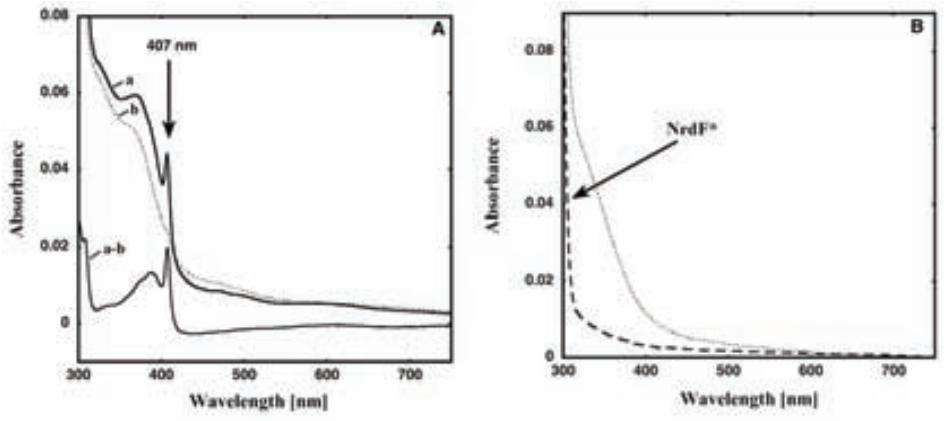

Figure 25. A. a. UV-vis spectra of *S. pyogenes* NrdF protein. The arrow indicates the tyrosyl radical. **b.** Dotted line indicates the HU-treated sample and **a-b.** shows subtraction of the HU-treated spectrum from the purified NrdF spectrum. **B.** UV-vis spectra of *S. pyogenes* NrdF* protein upon purification (dashed line) and after addition of 2 Fe/monomer.

Addition of the radical scavenger hydroxyurea to reduce the tyrosyl radical gave the spectrum shown in figure 25A (dotted line), where it is shown that the diiron site remained essentially unchanged. Subtraction of the endpoint spectrum gives the tyrosyl radical spectrum, (Fig. 25A, lowest spectrum) with the sharp peak at 407 nm. Using the extinction coefficient e=3.25 $mM^{-1}cm^{-1}$ in the subtracted spectra or using the dropline procedure, we calculated, for the best preparation, a radical content of 0.51 per monomer, in good agreement with the 0.57 value determined by EPR. It is not uncommon that RNRs purify with suboptimal or no iron content and, consequently, low radical, when overexpressed in *E. coli*. Hence, we added ferrous ions to the proteins to investigate if we could increase the yield of the diiron-radical site. Addition of ferrous ions, however, did not increase the radical yield of NrdF in the radical containing samples.

Another way to characterize tyrosyl radicals is by determining their sensitivity to HU. It was found that *S. pyogenes* reacted with a considerably lower rate than *E. coli* NrdB, k_1 0.00195 s^{-1} with 60 mM HU compared to 0.00828 s^{-1} for *E. coli* with 15 mM HU at 25 °C (i.e. at least 8 times less sensitive than the *E. coli* enzyme) (data not shown).

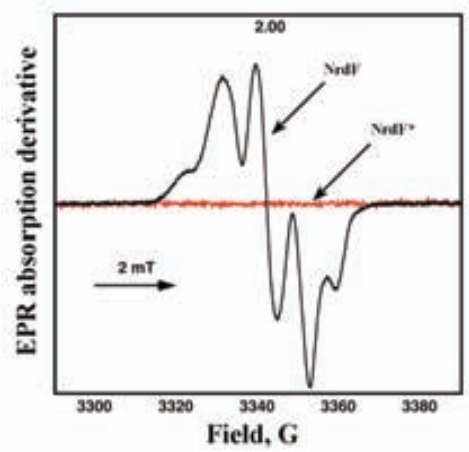

Figure 26. First-derivative EPR spectra of NrdF (black) and NrdF* (red)

On the other hand, purified NrdF* showed no prominent features in light absorption spectra (Fig. 25B, dashed line) as well as a flat EPR signal, *i.e.* it had no diiron-tyrosyl radical site (Fig. 26, red line). Analysis of the NrdF* iron content showed approximately 0,051 Fe/monomer (Table V), in good agreement with the light absorption and EPR signals observed. Addition of 2 Fe/monomer gave a spectrum without radical and without the characteristics of the diiron site observed in the NrdF protein, but with absorption in the 325-450 nm region similar to what has been observed in iron ligand mutants in *E. coli* NrdB (Fig. 25B, dotted line) (e≈1800 $M^{-1}cm^{-1}$) (Andersson *et al.*, 2004; Voegtli *et al.*, 2003). Iron analysis of desalted samples gave values around 0.47 Fe/monomer.

Table V. Iron and radical content of *S. pyogenes* NrdF and NrdF* before and after iron reconstitution

	Fe / Monomer (nmols)	**Tyr · / Monomer**
NrdF	1.159	0.5
$NrdF_R$	2.108	0.5
NrdF*	0.051	ND[a]
$NrdF_R$*	0.47	ND[a]

[a]ND, Not detected

R, Iron reconstituted

In order to investigate the nature of the metal site, we ran EPR measurements at 10-20 K. An antiferromagnetically coupled diiron site would be EPR silent, a Fe(III)/Fe(II) site

would give a typical signal in the g 1.7 region, a high spin Fe (III) could be observed at g 6 and 9 if the coordination of the iron ligands were axial, at g 4.3 if the coordination were rhombic. The only prominent feature observed was the g 4.3 resonance after subtraction of the cavity and buffer background (data not shown).

3.2.5 - Enzymatic activity

When assayed for enzymatic activity, all four proteins alone lacked activity. The NrdE and NrdF proteins purified from the *nrdHEF* system, however, showed proportional dCDP formation with one protein in excess and increasing amounts of its partner, with specific activities, in the presence of dATP as an allosteric effector, of 169 and 45 nmol/min/mg for NrdF and NrdE respectively (Table VI).

Activity was also dependent on the addition of magnesium and DTT at an optimal pH of 8.0 and required CDP as a substrate with a K_M of 0.34 mM (Fig. 27A). CDP reduction was strongly stimulated by dATP as an effector and not by ATP. Optimal activity with dATP was obtained at nucleotide concentrations as low as 0.01 mM, and no significant inhibition was seen even with 2 mM dATP. When ATP was used, concentrations up to 2 mM were not enough to achieve optimal activity. From figure 27B, an apparent K_L of 0.0011 mM for dATP can be calculated. No significant gain in specific activity was detected for the iron reconstituted NrdF protein, in agreement with the data provided by EPR and UV-visible spectroscopy.

Table VI. Specific activity (nmol mg^{-1} min^{-1}) of the *S. pyogenes* class Ib proteins under all possible combinations

	NrdE	**NrdF**	**NrdE***	**NrdF***
NrdE		**45**		ND[a]
NrdF	**169**		ND[a]	
NrdE*		ND[a]		ND[a]
NrdF*	ND[a]		ND[a]	

[a]ND, Not detected

The NrdE* and NrdF* proteins, on the other hand, had no activity when assayed together as above, nor when the NrdF* was paired with NrdE, which agrees with the lack of an organic radical in NrdF*. Presumably the altered residues within the iron binding center of this protein impair radical formation and render the NrdF* inactive.

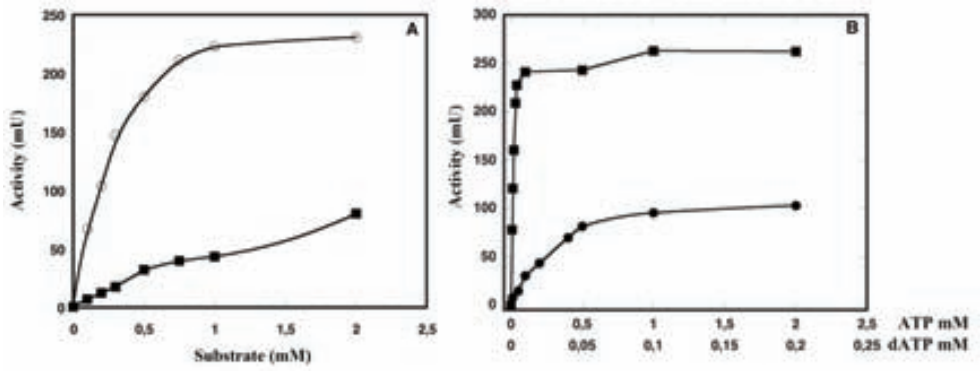

Figure 27. A. Comparison of CDP and CTP as substrates for the NrdF/NrdE reductase. Assays were made under standard conditions with 1.2 μM of NrdE and 5 μM of NrdF (CDP, ○; CTP, ■). mU, milliunits. **B.** Effect of ATP and dATP on CDP reduction. Incubation was with 1.2 μM of NrdE and 5 μM of NrdF, replacing the standard concentration of 0.2 mM dATP by the indicated concentrations of either ATP (●) or dATP (■). mU, milliunits

Some activity was expected, however, when combining the NrdE* and the NrdF proteins, since NrdF harbours a functional tyrosyl radical, but again we could not detect formation of reduced CDP from this pairing (Table VI).

Although no activity was ever detected when the assay included either NrdF* or NrdE* in combination with the corresponding partner from the *nrdHEF* system, it was not clear if the holoenzyme was ever formed. A competitive inhibition assay was developed in order to detect protein interaction between subunits belonging to each system. Increasing amounts of either NrdF or NrdE were assayed against fixed amounts of both its natural counterpart and its homologue in the non-working operon. As shown in figure 28, concentrations up to 2 μM of NrdF* or NrdE* had no inhibitory effect on NrdF/NrdE holoenzyme formation.

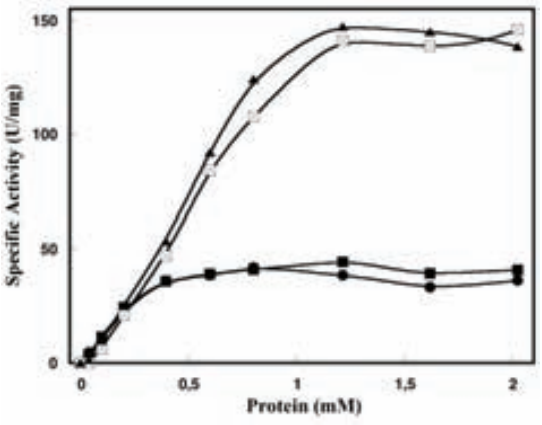

Figure 28. Competitive inhibition assay on both NrdE and NrdF. Incubations were under standard conditions with increasing concentrations of NrdF in the presence of 0.6 μM NrdE (●) and 2 μM of NrdF* (■), or with increasing concentrations of NrdE in the presence of 0.6 μM NrdF (☒) and 2 μM of NrdE* (▲)

3.2.6 - *In vivo* complementation

Despite the disappointing results obtained *in vitro* concerning the *nrdFIE** encoded enzyme, we decided to test whether this operon encoded an *in vivo* functional enzyme in a heterologous complementation assay. In order to do so we used the *E. coli* IG101 strain, which is a $nrdA_{ts}B1$/*nrdH*::Spc double mutant unable to reduce ribonucleotides aerobically at 42ºC unless a functional ribonucleotide reductase is provided to complement the temperature sensitive phenotype. The *nrdFIE** entire sequence was cloned into the pBAD18 vector under control of the *E. coli* arabinose promoter (P_{BAD}), an on/off switch promoter repressed by glucose and induced by arabinose, generating plasmid p18-FIE*Spyo, which was then transformed into the *E. coli* IG101 strain. Complementation was determined by plating serial dilutions of liquid cultures on LB agar plates supplemented with either 0.3 % L-arabinose or 0.3 % D-glucose at both 30 and 42ºC. As a positive control for this assay, we used the *Salmonella typhimurium nrdHIEF* entire sequence and the *S. pyogenes nrdHEF* operon cloned into the pBAD18 vector as well (which yielded plasmids p18-HIEFSal and p18-HEFSpyo, respectively).

Unexpectedly, the IG101 strain transformed with p18-HEFSpyo was not able to grow at 42ºC either in the presence of arabinose or glucose. Surprisingly, as many as $1.56x10^8$ cfu/ml were observed after 24-48 hours at 42ºC in those plates supplemented with arabinose, but not in those with glucose, for the IG101 strain transformed with p18-FIE*Spyo, indicating that the *nrdFIE** cluster was capable of overcoming the temperature-sensitive phenotype (Table VII). The same results were obtained when using the low-copy number pBAD33

instead of pBAD18, a similar vector bearing a distinct origin of replication and antibiotic resistance (data not shown).

Neither the pBAD18 nor the pBAD33 alone were able to support growth of the IG101 strain at the restrictive temperature.

Our next step was to determine if all genes within the *nrdFIE** operon were equally necessary for the observed *in vivo* complementation. A set of pBAD18/pBAD33 vectors were constructed so that every single gene within the *nrdFIE** operon and every possible pairwise combination of them could be evaluated. As shown in table VII, no complementation was detected when analysing each gene independently, which was already expected since class Ib ribonucleotide reduction requires, at least, the assembly of the large and the small subunits.

Surprisingly, we were also unable to detect growth at the restrictive temperature when any of the three *nrdFIE** genes were missing (Table VII), although heterologous complementation could be restored by providing the missing gene *in trans*.

These results indicate the compulsory requirement of both large and small subunits to form a functional holoenzyme but, above all, they reveal the essential role of the *nrdI** gene for *in vivo* activity.

In view of these results, both the *S. pyogenes nrdI** and *nrdI2* genes cloned into pBAD33 were co-transformed with the *nrdHEF* operon into the IG101 strain and assayed at 42ºC in arabinose and glucose LB plates. However, and as shown in table VII, addition of any of the *S. pyogenes nrdI* genes did not suffice to overcome the temperature-sensitive phenotype.

We then decided to check whether an *nrdI* gene from the closely related bacterium *Streptococcus pneumoniae*, whose genome harbours an *nrdHEF* operon as well as an isolated *nrdI2* gene, but not a *nrdFIE* operon, could restore complementation when combined with the *S. pyogenes nrdHEF*. Plasmids p18-HEFSpn and p33-I2Spn were generated by cloning the *nrdHEF* and *nrdI2* genes from *S. pneumoniae* into the pBAD vectors, as before, and were used in an heterologous complementation assay over the IG101 strain.

When assayed alone, the *S. pneumoniae nrdHEF* operon did not complement for the lack of an active RNR at 42ºC. On addition of the NrdI2Spn gene, however, growth of the IG101 strain at 42ºC in arabinose LB plates (but not in glucose) indicated complementation of the temperature-sensitive phenotype and, thus, the presence of a functional RNR *in vivo*.

These results also strengthen our previous observation concerning the essentiality of an *nrdI* gene for *in vivo* ribonucleotide reduction.

Moreover, the IG101 strain was also capable of growing at 42ºC when the *nrdHEF* operon from *S. pyogenes* was transformed together with the *nrdI2* gene obtained from *S. pneumoniae*, although the plating efficiency decreased considerably (Table VII).

Table VII. CFU/ml and plating efficiencies for pBAD constructs in the heterologous complementation assay

pBAD constructs	Mean CFU/ml ± SD at 42ºC 0.3 % arabinose	Mean plating efficiency ± SD[a]
E. coli IG101	0	0
pBAD18	0	0
pBAD33	0	0
p18-HIEF Sal	**1.56 x10^{8}** ± 9.60 x10^{5}	**0.214** ± 1.31 x10^{-3}
p18-HEF Spyo	0	0
p18-FIE*Spyo	**1.56 x10^{8}** ± 8.10 x10^{5}	**0.214** ± 1.11 x10^{-3}
p33-F*Spyo	0	0
p33-I*Spyo	0	0
p33-E*Spyo	0	0
p33-I2 Spyo	0	0
p18-FI*Spyo	0	0
p18-FE*Spyo	0	0
p18-IE*Spyo	0	0
p18-FI*Spyo / **p33-E***Spyo	**1.56 x10^{8}** ± 5.69 x10^{5}	**0.214** ± 7.78 x10^{-4}
p18-FE*Spyo / **p33-I***Spyo	**1.56 x10^{8}** ± 4.24 x10^{5}	**0.213** ± 5.80 x10^{-4}
p18-IE*Spyo / **p33-F***Spyo	**1.54 x10^{8}** ± 7.78 x10^{5}	**0.211** ± 1.06 x10^{-3}
p18-HEF Spyo / **p33-I2**Spyo	0	0
p18-HEF Spyo / **p33-I***Spyo	0	0
p18-HEF Spn	0	0
p33-I2 Spn	0	0
p18-HEF Spn / **p33-I2**Spn	**1.55 x10^{8}** ± 9.74 x10^{5}	**0.212** ± 1.33 x10^{-3}
p18-HEF Spn / **p33-I2**Spyo	0	0
p18-HEF Spn / **p33-I***Spyo	0	0
p18-HEF Spyo / **p33-I2**Spn	**9.66 x10^{4}** ± 3.21 x10^{5}	**0.00013** ± 4.39 x10^{-6}

[a] Plating efficiencies were determined as the fraction of CFU/ml under restrictive conditions compared to the number of CFU/ml under non-restrictive conditions. Values are based on results of six independent experiments

3.3 - Discussion

Examples of prokaryotic genomes displaying multiplicity of RNRs are easily found in almost every group of bacteria (Torrents *et al.*, 2002). The concurrence of an aerobic enzyme together with an anaerobic one in facultative microorganisms simply reflects a metabolic need, and in those species bearing combinations of RNR classes that can be used under the same environmental conditions (such as class I+II, class Ia+Ib, or class II+III), it has been proposed that one of the enzymes might be needed to support normal cell growth while the other would participate in maintaining a pool of dNTPs for DNA repair and growth recovery (Borovok *et al.*, 2002; Borovok *et al.*, 2004; Dawes *et al.*, 2003; Monje-Casas *et al.*, 2001; Smalley *et al.*, 2002; Torrents *et al.*, 2005a). It is tempting to speculate about regulatory mechanisms that would vary the amounts and activities of different RNRs in pathogenic bacteria to enhance virulence, but up to date there are no evidences of such mechanisms.

The genome of *S. pyogenes* is unique in the sense that it contains two operons encoding two different RNRs of the same class. Moreover, only three genes are found within each of these operons (instead of four, as would be expected for a class Ib system) since the *nrdHEF* cluster lacks the *nrdI* gene and *nrdFIE** lacks *nrdH*. It is worth noticing that a second *nrdI* gene (*nrdI2*) is found elsewhere within the *S. pyogenes* chromosome.

This work addresses class Ib duplicity in *S. pyogenes* from a functional point of view, in an attempt to elucidate which enzyme is used to support cell growth.

RT-linkage PCR shows that both *nrd* clusters are simultaneously transcribed, each originating a single continuous message and, therefore, constituting two independent polycistronic operons. They are also likely to be translationally active, because transcription and translation are coupled processes in prokaryotes. Besides, western blotting against rabbit-raised *nrdF* antibodies demonstrated the simultaneous presence of both *nrdF* and *nrdF** products within this microorganism (data not shown).

Attempts to obtain chromosomal mutants either by insertional or recombinant inactivation in all class Ib *nrd* genes were unsuccessful, which was initially attributed to the essentiality of these genes. However, when we used the same mutagenic strategies to obtain a

chromosomal mutant of the previously described non-essential *covR* gene (Federle *et al.*, 1999), mutant colonies were recovered at extremely low frequencies ($1x10^{-8}$), therefore, essentiality of the *S. pyogenes* class Ib *nrd* genes could not be asserted on the basis of these results.

***In vitro* biochemical characterization**

The β subunit (NrdF) from the *nrdHEF* cluster behaves as a normal class Ib RNR in the biochemical and biophysical experiments. The radical content of one radical/dimer is normally observed in active preparations of class I RNRs, despite the theoretical maximum value of two. Also the extinction coefficient at 370 nm for the active NrdF in figure 25A is calculated to 4270 $cm^{-1}M^{-1}$, which indicates a fully occupied diiron site compared to the *E. coli* NrdB, determined to 4350 $cm^{-1}M^{-1}$ (Petersson *et al.*, 1980). The inability to increase the diiron radical content by addition of ferrous ions to less active samples indicates that the irons remain bound to the protein and need to be reduced or removed to regenerate a fully active site, similar to the *E. coli* NrdB case (Petersson *et al.*, 1980).

NrdF also proved capable of reducing ribonucleotides when assayed *in vitro* in the presence of its corresponding α subunit. Specific activities for this system were within the range of those described in other class Ib systems such as *M. tuberculosis* (Yang *et al.*, 1997) or *C. ammoniagenes* (Fieschi *et al.*, 1998). The *nrdHEF* encoded enzyme also displays dATP insensitivity, which is a common feature in class Ib enzymes since their large subunit lacks 50 to 60 amino-terminal residues which in class Ia enzymes constitute the ATP/dATP allosteric activity site (Jordan & Reichard, 1998). Alignment of both NrdE proteins from *S. pyogenes* together with previously described class Ib and Ia large subunits shows that they lack approximately 50 residues at their amino terminal end. Collectively these results show that the *nrdHEF* system behaves as a functional class Ib enzyme *in vitro*.

More interesting is the failure to *in vitro* detect enzymatic activity nor the presence of an diiron-tyrosyl radical when using the purified proteins from the *nrdFIE** cluster. The NrdF* protein does not display any RNR characteristics but a lower extinction coefficient of ca 1800 $M^{-1}cm^{-1}$ at 350 nm. It seems clear from the g 4.3 EPR signal and light absorption analysis that, although the reconstitution protocol showed some iron bound to the protein, the substituted iron ligands do not allow proper coordination of an iron centre capable of generating a tyrosyl radical under the conditions tested.

In vivo heterologous complementation and *nrdI* essentiality

Our *in vivo* results however, showed a complete different picture. In our heterologous complementation assay the *nrdFIE** operon turned out to be functional while the *nrdHEF* cluster failed to support growth of the *E. coli* strain at the restrictive temperature.

It would appear that the *nrdFIE** operon, despite being biochemically inactive *in vitro*, encodes a functional enzyme after all. Moreover, *in vivo* complementation by *nrdFIE** was only achieved when all three genes were simultaneously present, although not necessarily in *cis*, thus indicating the compulsory requirement for the *nrdI** gene product. In a similar way, the *S. pneumoniae nrdHEF* operon was only able to complement the *E. coli* temperature-sensitive phenotype upon addition of its own *nrdI* gene.

Essentiality of NrdI proteins is also supported by the fact that an *nrdI*-like gene can be found in every class Ib containing microorganism (http://rnrdb.molbio.su.se/) and preliminary assays in our laboratory with the *Salmonella typhimurium* class Ib RNR indicate that this gene too is required for *in vivo* class Ib ribonucleotide reduction in this microorganism (I. Sala unpublished results). The *Bacillus subtilis nrdI* gene has also been shown to be essential (Kobayashi *et al.*, 2003; Scotti *et al.*, 1996).

In view of these results, an explanation for the lack of *in vivo* activity of the *S. pyogenes nrdHEF* operon is straight forward: since *nrdI** seems to be essential for *in vivo* complementation, an *nrdI* gene must be added to the assay. To our surprise, however, addition of either *nrdI** or *nrdI2* from *S. pyogenes* did not render *nrdHEF* functional *in vivo*, but it did addition of the *S. pneumoniae* homologue *nrdI2*.

Since very little is known about NrdI-like proteins, it is hard to provide a solid explanation for this result. Protein comparison between the *S. pyogenes* NrdI* and streptococcal NrdI2 proteins showed that they are distant relatives (36% similarity), and such dissimilarity may impair the functional NrdI* from contributing to the *in vivo* ribonucleotide reduction carried out by NrdHEF.

On the other hand, sequence comparison between streptococcal NrdI2 proteins showed that, although they all displayed a high degree of similarity, two different groupings could be distinguished according to small differences in their primary sequences (Fig. 29). The first group comprised the NrdI2 proteins from *S. pneumoniae*, *S. mutans* and *S. suis*, being this protein the only NrdI-like protein present within their genomes, while the second

group included those from *S. pyogenes*, *S. agalactiae* and *S. equi*, all of which turned out to posses an *nrdFIE* cluster as well. As shown in figure 29, clustering of these sequences into two separate groups is mainly due to a 3 bp indel plus approximately ten substituted residues that are conserved in at least one of the groups.

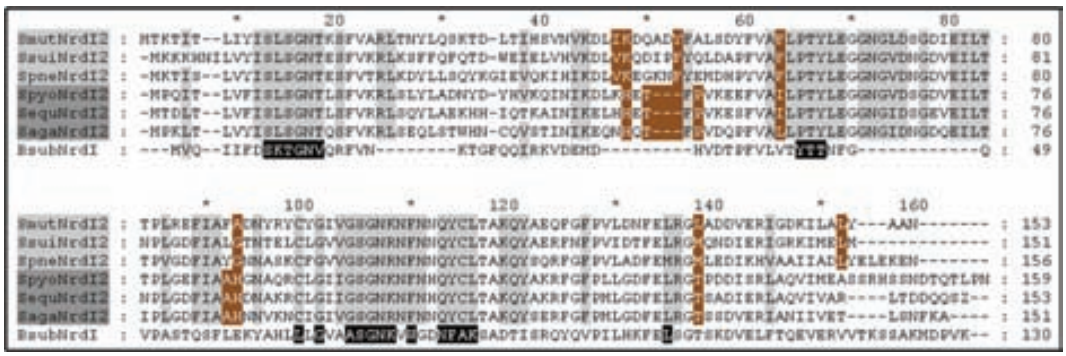

Figure 29. Clustal W alignment of deduced aminoacid fragments of the *nrdI* gene. All sequences were retrieved from the NCBI database at http://www.ncbi.nlm.nih.gov/ or from the Sanger Institute at http://www.sanger.ac.uk/. **Smut**: *Streptococcus mutans*; **Ssui**: *Streptococcus suis*; **Spne**: *Streptococcus pneumoniae*; **Spyo**: *Streptococcus pyogenes*; **Sequ**: *Streptococcus equi*; **Saga**: *Streptococcus agalactiae*; and **Bsub**: *Bacillus subtilis*;. Shading of the sequence names indicates common clustering. Those residues unique to each group are highlighted in brown. Conserved residues among all streptococci are highlighted in grey and the FMN binding residues in *B. subtilis* are shown in black

Interestingly, a *nrdFIE* cluster of genes displaying a high degree of similarity (87%, 57% and 76% for NrdF*, NrdI and NrdE respectively, at the protein level) to the streptococcal *nrdFIE** operon can also be found within all species of the *Mycoplasma* genus (Fig. 30A). Furthermore, and in addition to the striking sequence similarity between them, the three iron binding substituted residues described for the NrdF* protein are also conserved in all *Mycoplasma* NrdF subunits (Fig. 30B).

Mycoplasma comprises a group of intracellular parasites that take advantage of their host in many metabolic routes. It is possible, however, to grow them on culture, although these are very fastidious organisms and a highly enriched medium must be used. Mycoplasmas are considered to be the simplest prokaryotes capable of self-replication and,

thus, their gene content is usually discussed in terms of what genes are needed to constitute the minimal self-replicating genome (Koonin, 2000).

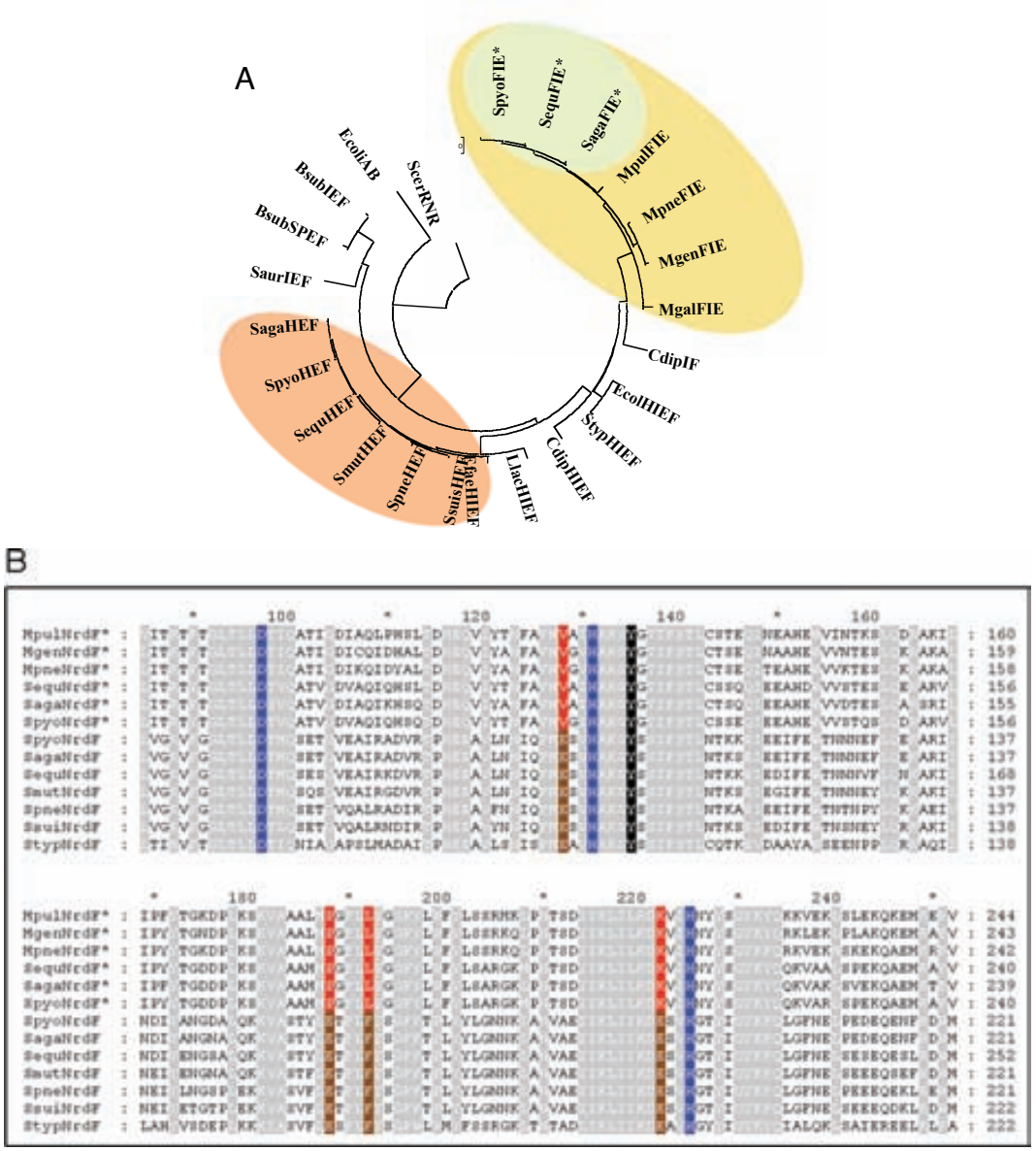

Figure 30. A. Phylogenetic tree showing the distribution of several class Ib operons. The differential clustering of streptococcal *nrdHEF* and *nrdFIE* operons and those from *Mycoplasma* is also shown. **Scer:** *Saccharomyces cerevisae*; **Bsub:** *Bacillus subtilis*; **BsubSP:** *Bacillus subtils* SPß prophage; **Saur:** *Staphylococcus aureus*; **Saga**: *Streptococcus agalactiae*; **Spyo**: *Streptococcus pyogenes*; **Sequ**: *Streptococcus equi*; **Smut**: *Streptococcus mutans*; **Spne**: *Streptococcus pneumoniae*; **Ssui**: *Streptococcus suis*; **Sthy**: *Salmonella typhimurium*; **Efae:** *Enterococcus faecalis*; **Llac:** *Lactococcus lactis*; **Cdip:** *Corynebacterium diphteriae*; **Styp:** *Salmonella typhimurium*; **Ecol:** *Escherichia coli*; **Mgal:** *Mycoplasma gallisepticum*; **Mgen:** *Mycoplasma genitalium*; **Mpne:** *Mycoplasma pneumoniae*; **Mpul:** *Mycoplasma pulmonis*. **B.** Clustal W alignment of deduced aminoacid

fragments of the *nrdF* gene. All sequences were retrieved from the NCBI database at http://www.ncbi.nlm.nih.gov/ or from the Sanger Institute at http://www.sanger.ac.uk/. Conserved residues are highlighted in grey, the conserved radical tyrosyl residue is shown is black and conserved iron binding residues are highlighted in blue. Substituted residues in *S. pyogenes*, *S. equi*, *S. agalactiae*, *M. pulmonis, M. genitalium* and *M. pneumoniae* are highlighted in red.

Although it is likely that *Mycoplasmas* uptake deoxyribonucleotides from their host, under the minimal-gene-set concept, and considering the fact that ribonucletotide reductases are essential enzymes and *nrdFIE* constitutes the only putative ribonucleotide reductase present within all *Mycoplasma* (with the sole exception of *M. penetrans*, which bears a class III RNR as well), one could argue in favour of an actual role in ribonucleotide reduction for the *Mycoplasma nrdFIE* encoded enzyme and, hence, for its streptococcal counterpart.

The striking similarities between the *nrdFIE* operons from both *Mycoplasma* and *S. pyogenes* leads us to suggest that this operon may have been acquired by an horizontal gene transfer event in order to compensate for the loss of *in vivo* functionality of the streptococcal *nrdHEF* system, mainly due to a sequence alteration within the NrdI2 protein that, either turned this protein non-functional, or diminished its activity to an extent that a new ribonucleotide reductase was needed. *S. pyogenes*, *S. agalactiae* and *S.equi* are close relatives, and it is likely that the horizontal transfer of *nrdFIE** occurred in a common ancestor who shared its niche with one of the several species of *Mycoplasma* (i.e.: *S. agalactiae-M. genitalium*) and then diverged into the three streptococcal species as we know them today.

We have not found any phagic signatures or mobile elements flanking the streptococcal *nrdFIE** genes and their GC% content was within the range described for the coding regions of Group A Streptococcal strains (Beres *et al.*, 2002; Ferretti *et al.*, 2001; Smoot *et al.*, 2002) (Table VIII). Likewise, the codon usage of all seven class Ib *nrd* genes was in agreement with that calculated for the *S. pyogenes* entire genome at the Codon Usage Database (http://www.kazusa.or.jp/codon/). *Mycoplasma* species, however, have been shown to utilize UGA as the tryptophan codon, which in most bacterial species (including the genus *Streptococcus*) is used as one of the three stop codons. We were surprised to note that all conserved tryptophan residues that in the *Mycoplasma nrdFIE* genes were encoded by UGA had been adapted in *S. pyogenes* to not cause a translational stop. This finding reinforces our suggestion that these genes were acquired to provide a vital function within *S. pyogenes*.

Table VIII. GC% content of *S. pyogenes nrd* genes

nrdHEF cluster	***nrdH***	***nrdE***	***nrdF***	***nrdI2***
GC% content[a]	34.24	37.9	36.14	46.58
*nrdFIE** cluster	***nrdF****	***nrdI****	***nrdE****	
GC% content[a]	43.29	41.71	45.48	
S. pyogenes strains	**SF370 M1**	**MGAS8232 M18**	**MGAS315 M3**	
GC% content	39.1[b]	38.6[c]	38.5[b]	

[a] Mean values calculated from the *nrd* genes of strains SF370, MGAS8232 and MGAS315
[b] Average GC% content of the protein-coding sequences
[c] Average GC% content of the complete genome

NrdI is a flavodoxin-like protein

In 2003 Joachimiak and co-workers submitted the three-dimensional structure of the NrdI protein from *Bacillus subtilis* (PDB: 1RLJ) (Wu *et al.*, 2003), although up to date a publication accompanying their results is still missing. This structural data, however, provided evidence that NrdI proteins contained a flavodoxin-like fold and, thus, they were classified as members of the flavodoxin NrdI family (Pfam accession: PF07972).

Flavodoxins are small electron transfer proteins that utilize a non-covalently bound flavin mononucleotide (FMN) as the redox active component (Sancho, 2006). In *E. coli*, both flavodoxin and NADPH:flavodoxin oxidoreductase have been shown to be essential for class III ribonucleotide reductase activation, where they provided a source of electrons which allowed reduction of the iron cluster (Mulliez *et al.*, 2001), and initial characterization of the class Ia enzyme clearly indicated the involvement of NADPH:flavodoxin oxidoreductase, flavin and an unidentified proteic fraction, in the reduction of the iron center (Fontecave *et al.*, 1989).

We suggest that NrdI is a flavodoxin like protein, which may exert a pivotal role in class Ib ribonucleotide reduction by providing the electrons needed to reduce the iron cluster and activate the radical. If this is to be true, class Ib operons would carry two specific electron donors, one providing reducing power to the large subunit (NrdH), and one supplying electrons to the smaller one (NrdI).

Sequence comparison between the *B. subtilis* NrdI and the streptococcal NrdI2 proteins apparently indicates that all residues involved in FMN binding are fully conserved

among all the streptococcal polypeptide chains (Fig. 29) thus, a better characterization of NrdI proteins is required to account for the apparent loss of functionality shown by the *S. pyogenes* NrdI2.

Nevertheless, an NrdI protein does not seem essential for *in vitro* activity when assaying the *nrdHEF* system and, in a similar way, all class Ib enzymes analyzed up to date have also been proven active *in vitro* without addition of a NrdI protein (Huque *et al.*, 2000; Jordan *et al.*, 1994b; Jordan *et al.*, 1996b; Torrents *et al.*, 2005b; Yang *et al.*, 1997). All of these enzymes, however, were also shown to contain an iron center bound to their small subunit. Furthermore, Jordan *et al*. (Jordan *et al.*, 1997) showed that the activity of the *S. typhimurium NrdEF* enzyme could be slightly stimulated by addition of a partially purified NrdI recombinant protein. In *S. pyogenes* however, addition of a crude extract obtained from the SF370 strain did not improve activity of either *nrdHEF* or *nrdFIE** (data not shown).

We believe that the three iron-binding substituted residues within NrdF* may account for a much more labile iron-binding site, making NrdI a compulsory requirement to generate an active holoenzyme. On the other hand, purified NrdF, which contains all six iron-binding conserved residues, already carried an iron center plus an organic radical and was less dependant on NrdI *in vitro*.

4. - Part II - *Escherichia coli* Class III RNR

4.1 - Brief Introduction

Escherichia coli is a facultative anaerobe whose genome contains the genes for two class I enzymes (Ia and Ib respectively) and one class III RNR. Oxygen (O_2) availability is one of the most important regulatory signals and various one or two-component sensor/regulator systems control the expression of aerobic and anaerobic metabolism.

The *arcA* (aerobic respiration control) gene, encoding a pleiotropic transcriptional regulator, belongs to a two component regulatory system. When activated anaerobically by the membrane sensor protein encoded by *arcB*, ArcA typically represses a number of operons of aerobic function (Lynch & Lin, 1996b) and activates some genes of anaerobic metabolism (Fu *et al.*, 1991; Park & Gunsalus, 1995). On the other hand, the transcriptional regulator encoded by the *fnr* gene (fumarate and nitrate reduction) is a pleiotropic one component regulator that activates transcription of some genes involved in anaerobic metabolism and represses the synthesis of some enzymes required for aerobic growth as well (Kiley & Beinert, 1998; Williams *et al.*, 1998). FNR contains a 4Fe-4S cluster that, when reduced under anaerobic conditions, induces FNR dimer formation and activates its DNA binding capacity.

Very little is known about RNR transcriptional regulation in response to environmental shifts, especially in those organisms with the ability to express two or more different RNR. Recently, Boston and Atlung (Boston & Atlung, 2003) demonstrated that expression of the *E. coli nrdDG* operon has an FNR-mediated oxygen-responsive regulation. Two putative FNR boxes upstream of the +1 transcript start site were also proposed. In this study we have analysed both the *in vivo* and *in vitro* interactions of these boxes with the FNR transcriptional regulator, in an attempt to elucidate their role in *nrdDG* activation.

4.2 - Results

4.2.1 - FNR and ArcA requirements for class III RNR expression

An *nrdDG-lacZ* transcriptional fusion containing 295 bp upstream of the *nrdD* start codon was constructed and inserted in the *E. coli* chromosome in single copy, as described in experimental procedures, to generate strain IG40 (Table I). Assay of β-galactosidase activities in this strain showed a 10-fold increase in LacZ levels when it was incubated anaerobically compared to the levels obtained under aerobic conditions, thus indicating the presence of a regulatory mechanism sensing variations in O_2 availability for this promoter (Fig. 31). In order to examine the effects of FNR and ArcA on both the aerobic and anaerobic expression of the *E. coli nrdDG* operon, Δ*fnr* and Δ*arcA* mutations were respectively transduced to strain IG40 to generate strains IG34 (Fnr^-), IG35 ($ArcA^-$) and IG36 ($Fnr^-/ArcA^-$).

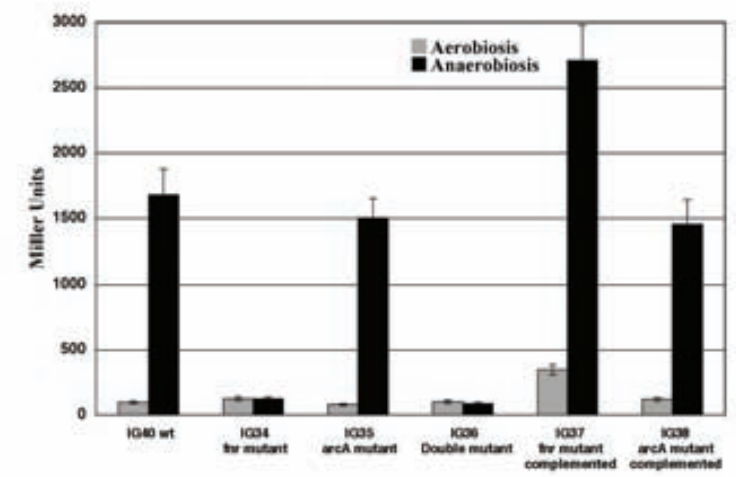

Figure 31. FNR-mediated regulation of *nrdDG*. β-Galactosidase levels (Miller Units) expressed from the *nrdD-lacZ* fusion in the: Wild-type strain (wt), *fnr* mutant strain, *arcA* mutant strain, *fnr/arcA* double mutant strain, *fnr* mutant strain complemented with an extrachromosomal *fnr* gene (pGS24), and *arcA* mutant strain complemented with an extrachromosomal *arcA* gene (pMW2) either grown aerobically □ or anaerobically ■. Results are expressed as the mean ±SEM of duplicates from three independent cultures

In the *fnr* mutant strain grown in the presence of O_2, β-galactosidase activities showed no difference with the wild-type strain. However, when this strain was incubated anaerobically, a 10-fold reduction in LacZ levels was detected relative to the fnr^+ strain (Fig. 31). To confirm that the mutation in *fnr* was responsible for reduced LacZ activities, we complemented the mutant strain with an expression plasmid containing the wild-type *fnr* gene. When assayed for β-galactosidase, the complemented strain recovered and surpassed the activity detected for the wild type strain grown anaerobically (Fig. 31).

On the other hand, β-galactosidase levels for the *arcA* mutant strain were as those found for the wild-type under both aerobiosis and anaerobiosis, even when complemented with an *arcA* containing plasmid, while the double mutant strain behaved as the fnr^- mutant (Fig. 31). At this point of our experimental research, a short paper containing very similar results was published by Boston and Atlung (Boston & Atlung, 2003), thus corroborating our data and providing further evidence that FNR was indeed responsible for anaerobic *nrdDG* activation.

4.2.2 - Binding of FNR* to the *nrdDG* promoter

Although ArcA consensus binding sites have been proposed from footprinting studies with several ArcA controlled promoters (Barcelona-Andres *et al.*, 2002; Drapal & Sawers, 1995; Lynch & Lin, 1996a; Shen & Gunsalus, 1997), we could not identify any region resembling an ArcA binding site within the *nrdDG* promoter region. However, Boston and Atlung described two putative FNR-boxes around -35 (FNR-1) and -65 (FNR-2) that displayed significant similarity with the consensus FNR-binding site, with only one or two mismatches (shown in boldface in Fig. 33) in either one of the palindromic half-sites.

In a preliminary search for specific FNR:DNA binding, gel retardation assays were performed to investigate whether FNR acts directly at the *nrdDG* promoter region. The same DNA fragment used to construct the *nrdD::lacZ* fusion was end-labelled to be used in gel retardation assays against purified FNR* protein (FNR-DA154). The DA154 substitution in this FNR protein has been shown to enhance dimer stability, thus providing an FNR form that is sufficiently active to perform gel retardation analysis under aerobic conditions, and has

been widely used to simplify *in vitro* analysis of FNR:DNA interactions (Kiley & Reznikoff, 1991; Lazazzera *et al.*, 1993; Ziegelhoffer & Kiley, 1995).

At the lowest protein concentration tested, a mobility shift band was readily detected, and as the concentration of FNR* increased, a second retardation band appeared in the gel (Fig. 32), thus indicating FNR binding to both putative boxes.

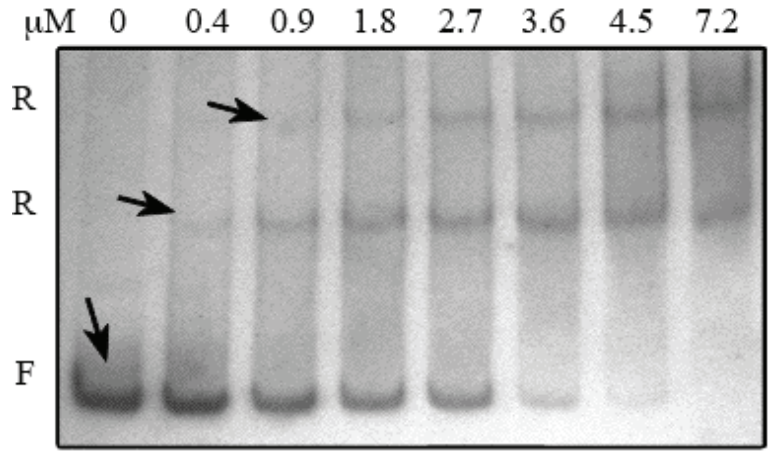

Figure 32. EMS assay with FNR* and the *nrdDG* promoter region. Arrows indicate positions of the free (F) and retarded (R) DNA. FNR* was added at the concentrations indicated above the lanes

In order to further demonstrate the direct involvement of these sequences in FNR recognition and binding, *in vitro* mutagenesis was used to independently mutagenize both FNR-2 and FNR-1 (see Experimental Prodedures), yielding two novel DNA fragments each containing only one unaltered FNR site (Fig. 33).

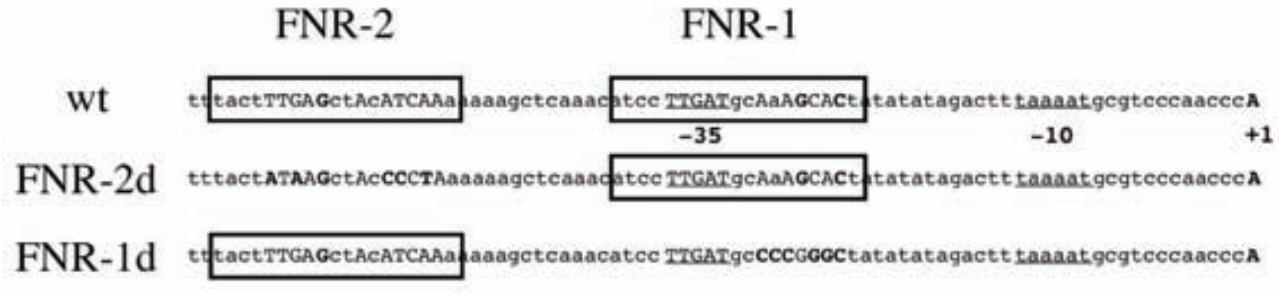

Figure 33. Sequence of the *nrdDG* promoter regions and the *nrdDFNR-2d* and *nrdDFNR-1d* mutated sequences. The FNR binding sites are boxed, and the –35 and –10 promoter regions are underlined. Bases fitting the consensus FNR sequence **TTGATNNANATCAA** (Green *et al.*, 2001) and the transcript start site (Boston & Atlung, 2003) are shown in uppercase. Mutated bases in the FNR binding sites are indicated in boldface

This DNA fragments were end-labelled and subsequently probed against FNR* in gel retardation assays. As shown in figure 34A, removal of the downstream FNR-1 site (FNR-1d) led to the formation of a single FNR*:*nrdD* complex, while no retardation at all could be observed when FNR-2 (FNR-2d) was the site being removed (Fig. 34B). Furthermore, the concentration of FNR* giving 50 % retardation of 1 μg *nrdD* (K_d) was calculated in all EMS assays and found to be the same for both the wild-type and the FNR-1d probe (K_d= 4.5 μM), so the absence of the downstream FNR site did not alter the binding affinity of the FNR-2 site. No shifted bands were observed when FNR* was incubated with a PCR probe lacking both the FNR-1 and FNR-2 sites (data not shown).

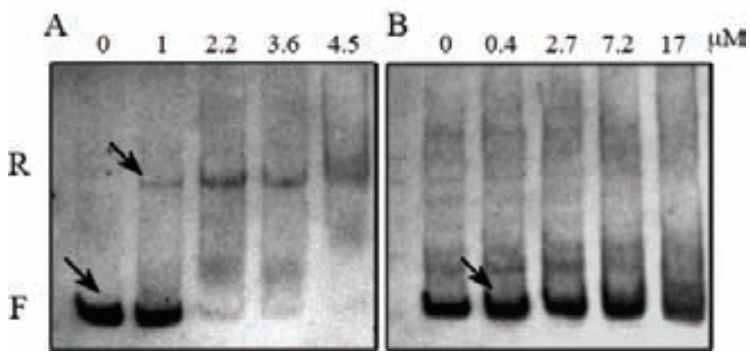

Figure 34. EMS assays with FNR* and both the *nrdDFNR-1d* (**A**) and *nrdDFNR-2d* (**B**) promoter regions. Arrows indicate positions of the free (F) and retarded (R) DNA. FNR* was added at the concentrations indicated above the lanes

The upstream promoter sequences of the *E. coli nrdAB* and *nrdEF* genes were also probed for FNR:DNA interaction but no retarded bands were observed (data not shown).

4.2.3 - *In vivo* usage of the FNR-2 and FNR-1 sites

Since modification of the putative FNR boxes affected FNR binding to the *nrdD* promoter sequence, we decided to test how such modifications might alter *in vivo* transcription as well. Therefore, chromosomal *lacZ* fusions to the defective promoter sequences were constructed as described in Experimental Procedures, generating strains IG41 (*nrdDFNR-2d::lacZ)* and IG42 (*nrdDFNR-1d::lacZ)*, and assayed for β-galactosidase under both aerobic and anaerobic growing conditions. As shown in figure 35, IG41 was unable to

activate β-galactosidase expression when grown anaerobically, thus behaving as the FNR mutant strain. On the other hand, lack of the downstream FNR-1 site did not impair IG42 from expressing β-galactosidase in an anaerobic environment, although expression was significantly reduced (two-fold) compared to the wild type fusion. These results corroborated those of the EMS assays.

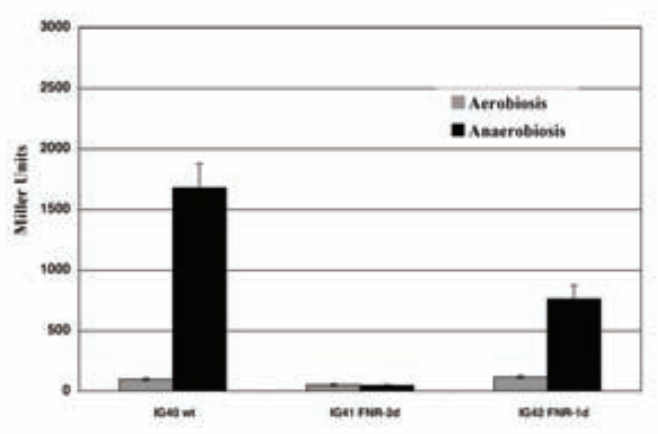

Figure 35. FNR-mediated regulation of *nrdDG*. β-galactosidase levels (Miller Units) expressed from the wild-type *nrdDG-lacZ* fusion, FNR-2 defective *nrdDFNR-2d-lacZ* fusion, and the FNR-1 defective *nrdDFNR-1d-lacZ* fusion either grown aerobically □ or anaerobically ■. Results are expressed as the mean ±SEM of duplicates from three independent cultures

4.3 - Discussion

There are just a few studies in the literature dealing with the genetic regulation of *nrd* genes, and most of them are focused on the aerobic classes (*nrdAB* and *nrdEF*), so the mechanisms that drive expression of the anaerobic enzyme are mainly unknown. However, since three different RNR classes are found within the *E. coli* genome, it seems reasonable that a mechanism switching on and off the expression of the *nrdDG* promoter in response to shifting oxygen concentrations may exist. Recently, Boston & Atlung (Boston & Atlung, 2003) showed that regulation of the *E. coli* anaerobic enzyme was FNR dependent, either through direct interaction of the protein to the promoter sequence or by means of a more complex pathway. The transcriptional fusions showed here between the *E. coli nrdDG* promoter and the *lacZ* gene corroborate their findings, and complementation studies of an *fnr* mutant strain provides further evidence for this FNR-dependent activation under anaerobic

growth. It also seems clear from our experiments that ArcA is not involved at all in *nrdDG* regulation, nor the *nrdAB* and *nrdEF* promoters seem to be FNR-dependent. However, although Boston & Atlung identified two putative FNR recognition sequences within the *nrdDG* promoter, they failed to provide direct evidence of their involvement in the anaerobic activation of the *E. coli* class III enzyme. The EMS assays presented in this work demonstrate a direct interaction between FNR* and the *nrdDG* promoter sequence. The fact that two shifted bands appeared in the gel also indicates involvement of both FNR sites in the FNR-dependent regulation. Furthermore, when faced with a DNA fragment lacking the downstream FNR-1 site, FNR* renders a single shifted band, while no retardation is observed at the tested protein concentrations when FNR-2 is not present. The need for the FNR-2 site in order to activate the anaerobic transcription from the *nrdDG* promoter was also found when transcriptional fusions with these defective promoter sequences were analysed. Unexpected were, however, the lower β-galactosidase levels obtained with the fusion lacking FNR-1.

In 1996, Garriga *et al.* (Garriga *et al.*, 1996) demonstrated that the *E. coli* anaerobic enzyme was essential for growth under strict anaerobiosis, but that an *nrdDG* null-mutant could also proliferate under microaerophilic conditions by overexpressing the *nrdA* gene (aerobic class Ia). Since it seems clear from our gel retardation experiments that both FNR sites display a differential affinity towards the FNR protein, being FNR-2 the site with the highest binding capacity, we suggest that these two sites are involved in fine tuning the shift from aerobic to anaerobic growth and we propose the following model to account for our results (Fig. 36).

It seems that maximal transcription from the *nrdDG* promoter requires the binding of two FNR dimers, with one dimer bound at each FNR site. Comparison of the $pO_{0.5}$ values of FNR and ArcA-regulated genes has suggested that the transition point from aerobic to anaerobic metabolism is found within the range of 1 to 5 millibars of O_2, and therefore, a coordinated substitution of the aerobic pathways by the anaerobic pathways must be achieved (Becker *et al.*, 1996). The FNR content in *E. coli* cells is known to be rather constant during aerobic and anaerobic growth. However, the proportion of the active reduced form has to increase gradually with decreasing pO_2 (Engel *et al.*, 1991). As the pO_2 decreases (microaerophilia), the amount of FNR that can bind DNA increases proportionally, and there is enough active FNR in the cell to bind to the high affinity FNR-2 site (which also displays

greater similarity to the FNR consensus sequence), but not to the FNR-1 site. Under this situation, expression from the *nrdDG* promoter is not maximal but, by overexpressing *nrdA*, the cell is able to proliferate. The lower β-galactosidase levels detected in our FNR-1d fusions would resemble this situation.

On the other hand, depletion of oxygen (strict anaerobiosis) renders class Ia RNR not functional, and the anaerobic enzyme becomes the only source of reduced ribonucleotides. At this point, the amount of active FNR is maximal and it binds to both FNR sites. Since FNR-1 is centered right on top of the –35 sequence, occupancy of this site might create closer contacts between RNA polimerase and the promoter sequence, achieving the higher levels of expression obtained with the wild type fusion.

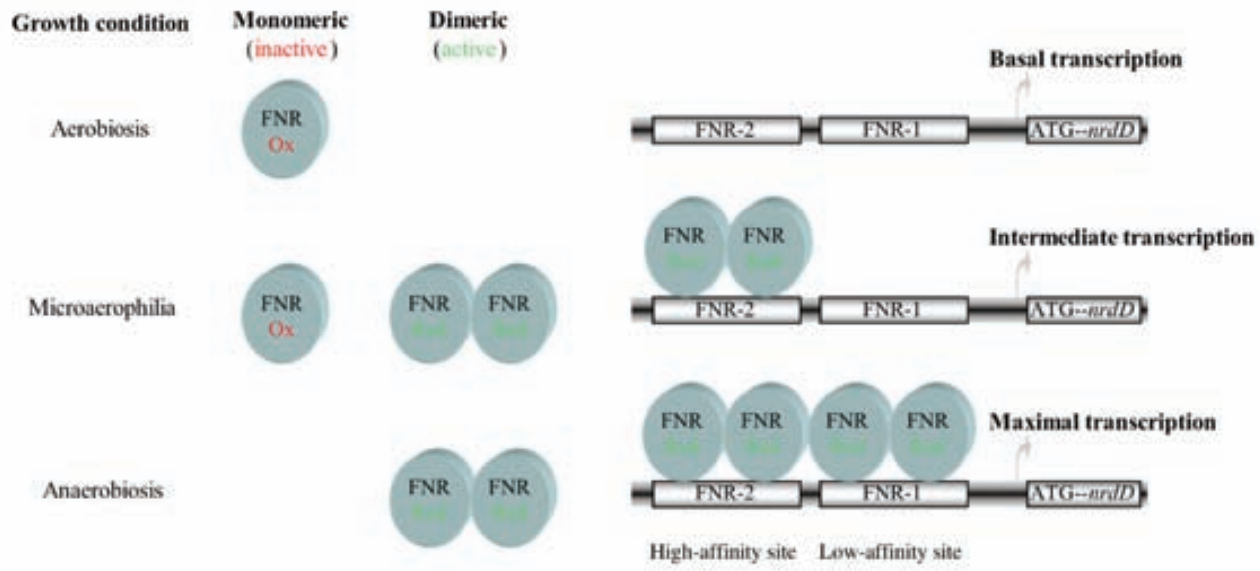

Figure 36. Schematic representing the FNR-dependent activation of *nrdDG* under shifting oxygen concentrations. **Ox:** Oxidized state; **Red:** Reduced state

Since we did not obtain any shifted bands at the tested protein concentrations with the FNR-2d probe, it is not clear if FNR binding to the FNR-1 site is due to cooperative binding with those FNR dimers bound to FNR-2, or if it is merely the result of a lower FNR-affinity. There is no doubt, however, about their synergic effect in the FNR-mediated activation of the *nrdDG* promoter.

This is the first time that direct evidence for the involvement of two FNR sites up-regulating an FNR-dependent promoter is provided, and further studies aiming to understand the mechanisms driving such regulation are under way.

5. - General Discussion and Future Aspects

For the last few years, our research group has been deeply concerned about the concurrence of RNRs within a single microorganism as well as about the mechanisms that govern the specific activation/repression of these enzymes under certain circumstances. It is appealing to believe that a redundant RNR enzyme might be of great importance under specific environmental conditions, such as during the course of an infection in pathogenic bacteria. In this respect, the study of class Ib redundancy in *Streptococcus pyogenes* was an ideal choice, since it is a human pathogen responsible for several life-threatening diseases. In fact, when we first noticed that one of the two class Ib operons in *S. pyogenes* contained a NrdF subunit (NrdF*, in this work) where three out of six iron-binding residues where not conserved, we thought that it might well be involved in supporting cell growth under circumstances where iron constituted a limiting factor, i.e. during an infective process.

The biochemical characterization of these two enzymes, however, turned out to be quite disappointing, as we were not able to detect radical formation or enzymatic activity when assaying the NrdFIE* enzyme. We eventually reached a point where we were seriously thinking about dumping the whole project and looking for a more suitable candidate. It was not until we realized that a similar (almost identical) *nrdFIE* operon constituted the only RNR enzyme within all *Mycoplasma* species that we decided to give it one more try. We therefore assayed the *in vivo* complementation of these operons over an heterologous microorganism such as *E. coli* and, to our surprise, *nrdFIE** turned out to encode a functional enzyme *in vivo*. Furthermore, the complementation assays led to a much more important discovery, NrdI essentiality. Previous experiments in our laboratory with the *S. typhimurium* NrdEF enzyme had already indicated a central role for *nrdI* genes in ribonucleotide reduction, but the results obtained with either the *nrdHEF* or the *nrdFIE** operons from *S. pyogenes* clearly demonstrate the compulsory requirement for an *nrdI* gene product.

Altogether, and although not yet conclusive, these results seem to indicate that class Ib redundancy in *S. pyogenes* may not be related to pathogenicity but to a lateral gene transfer event to compensate for the loss of functionality of an essential gene product.

Further work with *S. pyogenes* should investigate several relevant aspects that are not fully understood, such as the role of NrdI in ribonucleotide reduction (which should be approached from both a genetic and a biochemical point of view, although the hardships encountered in NrdI purification might be a serious drawback); the enzymatic activity and radical generation capacity of NrdFE* in combination with NrdI (which, of course, relies on the proper purification of NrdI*); and the improvement of a mutagenic procedure to obtain viable chromosomal mutants of the *nrdHEF* and *nrdI2* genes.

As more and more RNR enzymes from several organisms are being studied and no evidence of their involvement in pathogenicity is being provided, I seriously doubt that concurrence of RNRs plays a relevant role in such processes. I personally believe that the main function of the "secondary RNRs" found in many microorganisms is just to backup the "main RNRs" under certain circumstances, such as in DNA repair or oxidative stress.

As for the anaerobic FNR-dependent activation of the *E. coli* class III enzyme, it was sensible to believe that such an essential enzyme had to be modulated by one of the most important transcriptional regulators that govern aerobic/anaerobic transition. What it is indeed intriguing is the failure to detect FNR-binding to the promoter regions of either *nrdAB* or *nrdHIEF*. One would expect that the specific activation of the anaerobic enzyme were accompanied by the specific repression of its aerobic counterparts. In fact, *lacZ* fusions to the *S. typhimurium nrdAB* promoter region show a decrease in β-galactosidase levels when grown anaerobically (A. Panosa, personal communication), and a recent study in *S. typhimurium* also demonstrates enhanced transcriptional levels of *nrdAB* in a isogenic *fnr* mutant grown anaerobically when compared to the wild-type strain (Fink *et al.*, 2007).

However, it has also been reported in *E. coli* that a *nrdD*-null mutant shows increased NrdAB activity under microaerophilic conditions (Garriga *et al.*, 1996). It is then feasible that the increased anaerobic expression of *nrdAB* observed in *fnr* mutants responded to the lower *nrdDG* levels through the same unknown regulatory mechanism that modulates *nrd* expression in response to hydroxyurea, DNA damage, the absence of specific electron donors, and so on. This matter, nevertheless, deserves further investigation.

There is still a long way to go to fully understand the complex biochemistry and genetic regulation of ribonucleotide reductases, but we believe that the results provided within this work contribute to a better understanding of ribonucleotide reduction in bacteria.

6. – Conclusions

Part I

1. The *S. pyogenes nrdHEF* and *nrdFIE* genes constitute two independent polycistronic operons which are simultaneously transcribed
2. NrdF contains a diiron-tyrosyl radical site with 1.1 Fe/monomer and 0.5 radical/monomer
3. NrdE and NrdF form a biochemically functional holoenzyme *in vitro* and behave as a typical class Ib ribonucleotide reductase
4. NrdF* does not contain a diiron-tyrosyl radical site upon purification. *In vitro* reconstitution of the NrdF* diiron center generates 0.47 Fe/monomer in rhombic coordination
5. NrdF* and NrdE* are biochemically inactive *in vitro*, probably due to the lack of the diiron-tyrosyl radical site
6. The lack of enzymatic activity when crossing the large and small subunits of each system as well as the results from the competitive inhibition assay, seem to indicate that there are no protein interactions between subunits belonging to different systems.
7. The *nrdFIE** operon is active in the *in vivo* heterologous complementation assay and all three genes are essential to complement the temperature-sensitive phenotype. It is of especial interest the essentiality of the *nrdI** gene
8. The *nrdHEF* operons, either from *S. pyogenes* or from *S. pneumoniae*, are not functional in the *in vivo* heterologous complementation assay unless a functional NrdI protein is supplied. Neither NrdI* nor NrdI2 from *S. pyogenes* render these operons functional, but it does addition of the *nrdI gene* from *S. pneumoniae*
9. The primary sequence of the NrdI2 proteins from *S. pyogenes*, *S. agalactiae* and *S. equi* (all of which possess both a *nrdHEF* and a *nrdFIE** operon) is slightly different to that of the NrdI proteins from *S. pneumoniae, S. mutans* and *S. suis* (all of which only possess a *nrdHEF* operon).

10. The striking similarities between the *nrdFIE** operon from the three streptococcal species mentioned above and the *nrdFIE* genes from all species belonging to the genus *Mycoplasma* (where these are the only *nrd* genes present) lead us to suggest that an undetermined mutation in the streptococcal NrdI2 protein forced the acquisition (by some streptococcal species) of the *nrdFIE* operon present in *Mycoplasma* through a lateral gene transfer event

Part II

11. We have shown that there is indeed a differential expression of the *E. coli nrdDG* genes when comparing aerobic versus anaerobic growth

12. Expression of the *E. coli* anaerobic RNR genes is not mediated by the ArcA transcriptional regulator but it is activated during anaerobic growth in a FNR-dependent fashion

13. FNR-dependent activation of *nrdDG* relies on the specific binding of the FNR transcriptional regulator to two FNR-binding sites (FNR-2 and FNR-1) located upstream of the *nrdD* promoter. We could not detect, however, binding of FNR to the upstream promoter sequences of the *E. coli nrdAB* or *nrdEF* genes

14. The FNR-2 and FNR-1 binding sites display a differential affinity towards the FNR protein, being FNR-2 the site with the highest binding capacity

15. We suggest that such differential affinity is involved in fine-tuning *nrdDG* expression during the shift from aerobic to anaerobic growth

7. - Experimental Procedures

7.1 - Strains and Plasmids Used in this Work

Table IX. Strains used in this work

Strains	Genotype/characteristics	Source
Streptococcus pyogenes	*Streptococcus pyogenes Rosenbach* M1 SF370 (ATCC 700294)	ATCC
Streptococcus pneumoniae	*Streptococcus pneumoniae* (Klein) Chester R6 (ATCC BAA-255)	ATCC
***E. coli* DH5α**	*recA1 endA1 hsdR17 gyrA96 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR Φ80dlacZΔM15*	Clontech
***E. coli* BL21 (DE3)**	F^- *ompT gal [dcm][lon] hsdS$_B$ ($r_B^- m_B^-$)* with λ(DE3)	Novagen
***E. coli* KK450**	*nrdA$_{ts}$ nrdB1 thyA thr leu thi deo tonA lacy supE44 gyrA*	(Platz& Sjoberg, 1980)
***E. coli* MC1061Rif^R**	F^-, *araD139 Δ(ara-leu)7697 galE15 galK16 Δ(lac)X74 rpsL*(Str^R) *hsdR2 (r_K^-, m_K^+) mcrA mcrB1* Rif^R	Laboratory stock
***E. coli* MC1061λpir**	F^- *araD139 Δ(ara-leu)7697 galE15 galK16 Δ(lac)X74 rpsL(*Str^R*) hsdR2 (r_K^-, m_K^+) mcrA mcrB1 λpir*	Laboratory stock
***E. coli* S17-1λpir**	Tc^R Sm^R *recA thi pro hsdR$^-$M$^+$*RP4:2-Tc:Mu:Km Tn7*, λpir*	(de Lorenzo *et al.*, 1990)
***E. coli* RZ8480**	Δ*fnr lacZ*Δ145 *nar*G::MudI1734	(Lazazzera *et al.*, 1993)
***E. coli* ECL963**	φ(*cyd-lac) bla*$^+$ *cyd+ arcA2 zjj::*Tn*10*	(Fu *et al.*, 1991)
IG40	MC1061Rif^R *nrdDG-lacZ::Tn5Km-2*	This work
IG41	MC1061Rif^R *nrdDFNR-2d-lacZ::Tn5Km-2*	This work
IG42	MC1061Rif^R *nrdDFNR-1d-lacZ::Tn5Km-2*	This work

Strains	Genotype/characteristics	Source
IG34	IG40 Δ*fnr* (IG40 x RZ8480)	This work
IG35	IG40 *arcA2 zjj::*Tn*10* (IG40 x ECL963)	This work
IG36	IG34 *arcA2 zjj::*Tn*10* (IG34 x ECL963)	This work
IG37	IG34 with pGS24	This work
IG38	IG35 with pMW2	This work
IG100	DH5α *nrdH::*Spc	This work
IG101	KK450 *nrdH::*Spc (KK450 x IG100)	This work
P1vir Phage	Transducing phage	Laboratory stock

Table X. Plasmids used in this study

Plasmids	Genotype/characteristics	Source
pGEM®-T easy	A/T cloning vector	Promega
pUJ8	*trp'-'lacZ* promoter probe plasmid vector, *lacZ* fusions type I	(de Lorenzo *et al.*, 1990)
pUTminiTn5Km-2	Delivery plasmid for mini-Tn5 Km-2	(de Lorenzo *et al.*, 1990)
pMW2	pBR322 derivative containing the *arcA* gene	(Iuchi *et al.*, 1994)
pGS24	pBR322 derivative with *fnr* in a 1,64 kb *Hind*III-*Bam*HI fragment	(Shaw & Guest, 1982)
pGS771	pGEX-KG derivative with reconstructed fnr* in a 1156 bp NcoI-HindIII fragment	(Meng *et al.*, 1997)
pUC-Spc	*Streptococcus pyogenes* suicide vector	(Husmann *et al.*, 1997)
pET22b(+)	IPTG-dependent protein expression plasmid	Novagen
pET24a(+)	IPTG-dependent protein expression plasmid	Novagen
pBAD18Km	Arabinose/Glucose modulated protein expression plasmid	(Guzman *et al.*, 1995)
pBAD33Cm	Arabinose/Glucose modulated protein expression plasmid	(Guzman *et al.*, 1995)
pUC-Emut	pUC derivative containing an internal fragment of the *nrdE* gene	This work
pUC-Fmut	pUC derivative containing an internal fragment of the *nrdF* gene	This work
pUC-F*mut	pUC derivative containing an internal fragment of the *nrdF** gene	This work
pUC-I*mut	pUC derivative containing an internal fragment of the *nrdI** gene	This work
pUC-E*mut	pUC derivative containing an internal fragment of the *nrdE** gene	This work
pUC-I2mut	pUC derivative containing an internal fragment of the *nrdI2* gene	This work
pUC-CovRmut	pUC derivative containing an internal fragment of the *covR* gene	This work
p22-FSpyo	pET22b(+) derivative containing the *S. pyogenes nrdF* gene	This work

Plasmids	Genotype/characteristics	Source
p22-ESpyo	pET22b(+) derivative containing the *S. pyogenes nrdE* gene	This work
p22-E*Spyo	pET22b(+) derivative containing the *S. pyogenes nrdE** gene	This work
p24-F*Spyo	pET24a(+) derivative containing the *S. pyogenes nrdF**gene	This work
p18-HIEFSal	pBAD18Km derivative containing the *S. typhimurium nrdHIEF* operon	This work
p18-HEFSpyo	pBAD18Km derivative containing the *S. pyogenes nrdHEF* operon	This work
p18-FIE*Spyo	pBAD18Km derivative containing the *S. pyogenes nrdFIE** operon	This work
p33-F*Spyo	pBAD33Cm derivative containing the *S. pyogenes nrdF** gene	This work
p33-I*Spyo	pBAD33Cm derivative containing the *S. pyogenes nrdI** gene	This work
p33-E*Spyo	pBAD33Cm derivative containing the *S. pyogenes nrdE** gene	This work
p33-I2Spyo	pBAD33Cm derivative containing the *S. pyogenes nrdI2* gene	This work
p18-FI*Spyo	pBAD18Km derivative containing the *S. pyogenes nrdFI** genes	This work
p18-FE*Spyo	pBAD18Km derivative containing the *S. pyogenes nrdFE** genes	This work
p18-IE*Spyo	pBAD18Km derivative containing the *S. pyogenes nrdIE** genes	This work
p18-HEFSpn	pBAD18Km derivative containing the *S. pneumoniae nrdHEF* operon	This work
p33-I2Spn	pBAD33Cm derivative containing the *S. pneumoniae nrdI2* gene	This work
pIG72	pGEM®-T easy derivative containing a 479 bp fragment with the *nrdDG* promoter region	This work
pIG73	pGEM®-T easy derivative containing a 479 bp fragment with the *nrdDFNR-2d* promoter region	This work
pIG74	pGEM®-T easy derivative containing a 479 bp fragment with the *nrdDFNR-1d* promoter region	This work
pIG79	pUJ8 derivative containing the *nrdDG* promoter region fused to the *lacZ* gene	This work
pIG80	pUTminiTn5Km-2 derivative containing the *nrdDG* promoter region fused to the *lacZ* gene	This work

Plasmids	Genotype/characteristics	Source
pIG81	pUJ8 derivative containing the *nrdDFNR-2d* promoter region fused to the *lacZ* gene	This work
pIG82	pUTminiTn5Km-2 derivative containing the *nrdDFNR-2d* promoter region fused to the *lacZ* gene	This work
pIG83	pUJ8 derivative containing the *nrdDFNR-1d* promoter region fused to the *lacZ* gene	This work
pIG84	pUTminiTn5Km-2 derivative containing the *nrdDFNR-1d* promoter region fused to the *lacZ* gene	This work

7.2 - Primers Used in this Work

Table XI. Primers used in this study

Primer	Name	Sequence (5'→3')	Applications
1	Spyo/HEF-Hup	ACATATGATTACAGTATATTCC	Linkage RT-PCR
2	Spyo/HEF-Eintlw	GGAACGGTAATCCCTAGTGAC	Linkage RT-PCR
3	Spyo/HEF-Eintup	GGATGATGGGGAAGAAGTGGGCG	Linkage RT-PCR
4	Spyo/HEF-Fintlw	GAGGAGTGCGAACATCAGCACG	Linkage RT-PCR
5	Spyo/FIE-Fintup	GACCGTGCCCGTGTCTTGATTCC	Linkage RT-PCR
6	Spyo/FIE-Eintlw	CCAATTGATGGAGCGCAACTG	Linkage RT-PCR
7	Spyo/I2up	CATATGCCTCAGATTACCCTTG	Linkage RT-PCR
8	Spyo/I2lw	GGATCCATGGACTGTCTAGCTG	Linkage RT-PCR
9	Spyo/FIE-FupMut	GGATCCAGAAGTGTGGAACCG	*nrd* mutants
10	Spyo/FIE-FlwMut	GGATCCGTGAAGGGCGACTC	*nrd* mutants
11	Spyo/FIE-IupMut	GGATCCTCAGCGCATACCAG	*nrd* mutants
12	Spyo/FIE-IlwMut	GGATCCCGTGTGTGTGATGC	*nrd* mutants
13	Spyo/FIE-EupMut	GGATCCGCCTTGTGCCTCAC	*nrd* mutants
14	Spyo/FIE-EupMut	GGATCCTGTCGGAGCAACCG	*nrd* mutants
15	Spyo/HEF-EupMut	GGATCCGTCGTGGTGAATTGG	*nrd* mutants

Primer	Name	Sequence (5'→3')	Applications
16	Spyo/HEF-ElwMut	GGATCCTGGACTGCCATATTC	*nrd* mutants
17	Spyo/HEF-FupMut	GGATCCGAAGCTATTCGTGC	*nrd* mutants
18	Spyo/HEF-FlwMut	GGATCCCATCGTTTGCAGTATC	*nrd* mutants
19	Spyo/I2-upMut	GGATCCGTGGCAACACCCTTAG	*nrd* mutants
20	Spyo/I2-lwMut	GGATCCCCTCGCAGCTC	*nrd* mutants
21	Spyo/CovR-upMut	GGATCCAAGTCATTGTTGAGGTC	*nrd* mutants
22	Spyo/CovR-lwMut	GGATCCTGAGAGAAATCTCATCG	*nrd* mutants
23	Spyo/HEF-Fup	ACATATGACTACATATTATGAAGC	Protein overproduction
24	Spyo/HEF-Flw	AGGATCCTGAGCACTATTAGAGTC	Protein overproduction
25	Spyo/HEF-Eup	ACATATGAGTCTCAAAGATCTTGG	Protein overproduction
26	Spyo/HEF-Elw	AGGATCCTTACTTCTTAACCAATTAAATG	Protein overproduction
27	Spyo/FIE-Fup	ACATATGACCCAACATTATTATG	Protein overproduction
28	Spyo/FIE-Flw	AGGATCCATTTCTTAAAACTCCC	Protein overproduction
29	Spyo/FIE-Eup	ACATATGTCACAAACCAATGCG	Protein overproduction
30	Spyo/FIE-Elw	GGATCCGATAGGGGACACGAGAG	Protein overproduction
31	Spyo/RBS-HEFup	GAGCTCAAGGAGAACAAGACATGATTACAG	Heterologous complementation
32	Spyo/RBS-HEFlw	CTGCAGAGCACTATTAGAGTCC	Heterologous complementation
33	Spyo/RBS-FIEup	GAGCTCAAGGAGAGAGACAAATGACCCAAC	Heterologous complementation
34	Spyo/RBS-FIElw	CTGCAGGAGAGCTTTCTAGTAGC	Heterologous complementation
35	Spyo/RBS-I2up	GAGCTCAAGGAGAGACACTTATGCCTCAGA	Heterologous complementation

Primer	Name	Sequence (5'→3')	Applications
36	Spyo/RBS-I2lw	CTGCAGATGGACTGTCTAGCTG	Heterologous complementation
37	Spn/RBS-HEFup	TCTAGAAGGAGATTACACAATGGTAACCG	Heterologous complementation
38	Spn/RBS-HEFlw	CTGCAGTGAGAGTGATGGAGAATTAG	Heterologous complementation
39	Spn/RBS-I2up	TCTAGAAGGAGAGGTACATATGAAGAC	Heterologous complementation
40	Spn/RBS-I2lw	CTGCAGCATTGAGTATTAATTCTCC	Heterologous complementation
41	Spyo/RBS-I*up	GAGCTCAAGGAGATTAAGAAATGGCAGAGC	Heterologous complementation
42	Spyo/RBS-I*lw	CTGCAGGTGCATTTAATGAGAGG	Heterologous complementation
43	Spyo/RBS-E*up	GAGCTCAAGGAGCACACCCATGTCACAAACC	Heterologous complementation
44	Spyo/RBS-E*lw	CTGCAGATAGGGGACACGAGAG	Heterologous complementation
45	Ecol/Dproup	GAATTCTCGCTGTAGCGCCCG	PCR cloning and 5'-DIG end labelling
46	Ecol/Dprolw	GGATCCCATTGATATCCACCTGG	PCR cloning and 5'-DIG end labelling
47	Ecol/Dpro2dup	GCTTTTTACTATAAGCTACCCCTAAAAAAGCTC	Site-directed mutagenesis
48	Ecol/Dpro2dlw	GAGCTTTTTTAGGGGTAGCTTTTAGTAAAAAGC	Site-directed mutagenesis
49	Ecol/Dpro1dup	CCCGGGCTATATATAGACTTTAAAATGCG	Site-directed mutagenesis
50	Ecol/Dpro1dlw	GGATCCGCCCGGGGCATCAAGGATGTTTG	Site-directed mutagenesis

7.3 - Growth Media Used in this Work

LB medium (Luria Bertani) (Miller, 1972)

Triptone	10 g
Yeast Extract	5 g
NaCl	10 g

Dissolved in 1 L of deionized water and adjust the pH to 7.0 with 5 M NaOH

In order to prepare **solid LB agar** plates, add 17 g of agar

Autoclave (121ºC / 15 min)

LB medium soft agar

Triptone	10 g
Yeast Extract	5 g
Agar	7 g

Dissolved in 1 L of deionized water and autoclaved (121ºC / 15 min)

TB medium (Terrific Broth) (Tartoff & Hobbs, 1987)

Triptone	12 g
Yeast Extract	24 g
Glycerol	4 ml

Dissolved in 900 ml of deionized water and autoclaved (121ºC / 15 min)

Saline solution

KH_2PO_4	2.312 g
K_2HPO_4	16.32 g

Dissolved thoroughly in 100 ml of deionized water and autoclaved (121ºC / 15 min)

Allow both solutions to cool and combine them.

NaCl saline solution 0.9 %(Ringer)

NaCl	9 g

Dissolved in 1 L of deionized water and autoclaved (121ºC / 15 min)

Todd-Hewitt broth (Oxoid)

T-H broth	36.4 g

In order to prepare **solid T-H agar** plates, add 17 g of agar

Dissolved in 1 L of deionized water and autoclaved (121ºC / 15 min)

AB minimal medium

A solution

$(NH_4)_2SO_4$	20 g
Na_2HPO_4	60 g
KH_2PO_4	60 g
NaCl	30 g
Na_2SO_4	0.11 g

Dissolved in 1 L of deionized water and autoclaved (121ºC / 15 min)

B solution

$MgCl_2$ $6H_2O$	16 g in 500 ml of deionized water
$CaCl_2$	0.44 g in 250 ml of deionized water
$FeCl_3$ $6H_2O$	0.032 g in 250 ml of deionized water

Each salt solution was prepared and sterilized independently before mixing them into the final B solution.

In order to prepare 200 ml of AB minimal medium supplemented with glycerol nitrates and leucine the following sterilized solutions were mixed:

A solution	20 ml
B solution	5 ml
2 M glucose	1 ml
Glycerol	0.730 ml
2 M KNO_3	4 ml
1 mM Na2MO4 2H2O	0.2 ml
5 mg/ml Leucine	2 ml
Deionized water	Up to 200 ml

7.4 - Part I Specific Procedures

7.4.1 - Bacterial strains, plasmids and growth conditions

E. coli strains were routinely grown in Luria-Bertani (LB) at 15, 30, or 37ºC. *Streptococcus pyogenes Rosenbach* M1 (ATCC 700294, SF370) was grown in Todd-Hewitt broth (Oxoid) supplemented with 0.2 % yeast extract (THY) at 37ºC and in Columbia sheep blood agar plates (Biomerieux) whenever needed. *Streptococcus pneumoniae* (Klein) Chester R6 (ATCC BAA-255) was grown in Columbia sheep blood agar plates (Biomerieux) at 37ºC. Antibiotics and chromogenic substrates, when required, were included in the culture media or agar plates at the following concentrations for *E. coli*: 100 μg/ml carbamecillin, 100 μg/ml kanamycin, 34 μg/ml chloramphenicol and 30 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma); and for *S. pyogenes*: 100 μg/ml spectinomycin.

The *E. coli* strain IG101 (*$nrdA_{ts}$ nrdB1 thyA thr leu thi deo tonA lacy supE44 gyrA nrdH::*Spc) was constructed by introducing the *nrdH::Spc* allele (IG100, this laboratory) into KK450 (Platz & Sjoberg, 1980) by P1 transduction (see table IX).

7.4.2 - Sequence analysis and DNA manipulations

Genomic DNA from *S. pyogenes* was extracted as described in (Fieschi *et al.*, 1998). Electroporation of *S. pyogenes* was performed as described in (McLaughlin & Ferretti, 1995) and other general DNA manipulations were done by standard procedures (Sambrook *et al.*, 1989). DNA sequence determination was carried out using the BigDye Terminator Kit and an ABI prism 310 sequencer (Applied Biosystems) according to the manufacturer's specifications. All *nrd* sequences were searched in finished and unfinished NCBI (http://www.ncbi.nlm.nih.gov) microbial genomic databases. The ClustalW vs.1.8 (Higgins *et al.*, 1994) and GeneDoc vs.2.6.001 (Nicholas & Nicholas, 1997) programs were used to perform global alignments. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar *et al.*, 2004)

7.4.3 - RNA isolation

S. pyogenes was grown in 10 ml of THY until A_{600} was approximately 0.4. 10 ml cultures were centrifuged and resuspended in 200 μl of diethyl pyrocarbonate (DEPC, Sigma) treated distilled water and quick frozen in a dry ice bath. Pellets were thawed on ice and added to 2 ml FastPrep Blue tubes containing Zirconite/Silica beads, 500 μl of CPRS-Blue and 500 μl of acid phenol, as described by the manufacturer (Bio 101, La Jolla, Calif.). Bacteria were lysed with a FastPrep FP120 instrument (Bio 101, La Jolla, Calif.) at setting 6 for 11 s and immediately placed on ice after disruption. Samples were incubated at 65ºC for 10 min and centrifuged at 10.000 x g for 5 min at 4ºC. The upper aqueous phase was recovered and extracted with an equal volume of acid phenol heated to 65ºC. The phases were separated by centrifugation as above and the extraction was repeated with acid phenol:chloroform (1:1,v/v) and chloroform:isoamyl alcohol (24:1,v/v). The recovered aqueous phase was treated with 50 U of DNAse I (Ambion, Inc.) for 30 min at 37ºC and then 0.2 volumes of Inactivating Reagent (Ambion, Inc.) were added to eliminate the enzyme. RNA concentrations were determined by measuring the A_{260}. Samples were immediately stored at –80ºC. RT-PCR was performed as described in (Torrents *et al.*, 2003), and identical aliquots were processed in parallel without addition of RT, in order to ensure that residual chromosomal DNA was not being used as the template for PCR amplification.

7.4.4 - Construction of *S. pyogenes nrd* mutants

Plasmids for inactivation of the *S. pyogenes nrd* genes were constructed as follows. Fragments internal to the coding regions of the genes *nrdE, nrdF, nrdF*, nrdI*, nrdE** and *nrdI2* were amplified by PCR using wild-type chromosomal DNA as a template and primer pairs listed in table XI. The PCR-derived fragments were cloned into the pGEM-T easy vector and then were BamHI removed and ligated into the pUC-Spc suicide vector (Husmann *et al.*, 1997), to produce plasmids pUC-Emut, pUC-Fmut, pUC-F*mut, pUC-I*mut, pUC-E*mut and pUC-I2mut (Table X). These plasmids were introduced into *S. pyogenes* strain SF370 by electroporation and transformants were grown on THY agar with selection for spectinomycin.

As a positive control we used an internal fragment of the transcriptional regulator *covR* gene (Federle *et al.*, 1999).

7.4.5 - Cloning and expression

The *nrdF, nrdE, nrdF** and *nrdE** genes were amplified by PCR using primers pairs containing NdeI (upper primers) and BamHI (lower primers) restriction sites and *S. pyogenes* genomic DNA as a template (Table XI). The resulting fragments were purified and digested with NdeI and BamHI. The *nrdF, nrdE* and *nrdE** digested fragments were inserted into pET22b(+) (Novagen) cut with the same enzymes to give p22-FSpyo, p22-ESpyo and p22-E*Spyo respectively (Table X). The *nrdF** digested fragment was inserted into pET24a(+) (Novagen) cut with the same enzymes to give p24-F*Spyo (Table X). All of the above constructs were verified by sequencing.

E. coli BL21 (DE3) (Novagen) cells transformed with one of the expression plasmids (p22-FSpyo, p22-ESpyo or p22-E*Spyo) were grown overnight in LB medium containing 100 µg/ml carbenicillin at 30 or 37ºC. This culture was then used to inoculate 5-liter flasks containing 2 liters of LB medium with the same concentration of antibiotic as above (1:100). Cells were then grown at 30ºC under vigorous shaking to an $A_{600} \approx 0.5$-0.6 and subsequently induced with 0.5 mM IPTG (final concentration) for four hours.

BL21 (DE3) transformed with plasmid p24-F*Spyo was grown overnight in LB medium containing 100 µg/ml kanamycin at 30ºC. This culture was then used to inoculate 5-liter flasks containing 2 liters of LB medium with the same concentration of antibiotic as above (1:100). Cells were then grown at 30ºC under vigorous shaking. When reaching an $A_{600} \approx 0.5$-0.6, the cultures were chilled to 15ºC before being induced with 0.3 mM IPTG final concentration and then grown for 15-17 hours at 15ºC.

After induction, cells were harvested by centrifugation and the pellets quickly frozen and stored at –80ºC.

7.4.6 - Protein purifications

Frozen pellets were disintegrated by X-press (Biox AB) and extracted in 50 mM Tris-HCl pH 8, 50 mM KCl, 5 mM DTT and 1 mM PMSF (Buffer L), after which nucleic

acids were precipitated by drop wise addition of streptomycin sulphate to a final concentration of 1%. The precipitate was removed by centrifugation and solid $(NH_4)_2SO_4$ was added to the supernatant to 45% saturation for NrdE and NrdE*, and to 60% saturation for NrdF and NrdF*. The precipitate was further recovered by centrifugation.

The NrdF and NrdF* pellets were dissolved in buffer L and desalted by dialysis against 2 liters of buffer L for 4-5 h. This solution was diluted with buffer L to 5 mg/ml protein concentration and loaded onto a DEAE Sepharose column (GE Healthcare) coupled to a FPLC machine (Biologic DuoFlow Systems, Bio-Rad). After a washing step with 140 ml of 0.15 M KCl in buffer L, elution was continued with a 0.15-0.6 M KCl linear gradient in buffer L in a total volume of 300 ml. Fractions checked by SDS-polyacrylamide gel electrophoresis containing the NrdF or NrdF* protein were pooled and concentrated by ultrafiltration on a centriplus YM-30 (Millipore) against buffer L and stored at –80ºC for further purification. The NrdF proteins eluted between 0.3-0.36 M KCl. The concentrated enzyme solution was loaded onto a Superdex™ 75 10/300 GL column (GE Healthcare) previously equilibrated against buffer L. Proteins were eluted against the same buffer at a constant flow rate of 0.4 ml/min. Fractions containing NrdF or NrdF* were pooled, concentrated and stored as above.

For the purification of the NrdE and NrdE* proteins, the ammonium sulphate precipitated pellets were dissolved in 50 mM Tris-HCl pH 8, 0.75 M ammonium sulphate (Buffer A) and loaded onto a HiLoad® 16/10 PhenylSepharose® High Performance column (GE Healthcare) equilibrated with the same solution. Elution was performed with a 0.75-0 M ammonium sulphate linear gradient in 50 mM Tris-HCl pH 8. NrdE and NrdE* eluted between 0.44-0.35 M and 0.03-0 M ammonium sulphate respectively. The enzyme containing fractions were pooled and concentrated in Centriplus YM-50 (Millipore) and then adsorbed to a 2 ml MonoQ-anion exchange column (GE Healthcare) equilibrated with buffer L. Proteins were eluted with 20 ml of a 0.05-0.5 M KCl linear gradient in buffer L at a flow-rate of 0.1 ml/min. NrdE eluted between 0.21-0.28 M KCl and NrdE* between 0.19-0.27 M KCl. Fractions of 0.4 ml were pooled and concentrated as above and then stored at –80ºC.

7.4.7 - Analytical methods and enzyme activity assays

Iron reconstitution assays were performed as described in (Huque *et al.*, 2000) and iron content was determined as described in (Sahlin *et al.*, 1990). Protein concentration was determined by the Bradford method (Bradford, 1976).

RNR activity was assayed in 50 µl mixtures containing 50 mM Tris-HCl pH 8, 0.2 mM dATP as an effector, 20 mM magnesium acetate and 30 mM DTT. The reaction was started by addition of [^{3}H]-CDP (specific activity, 10,000-20,000 cpm/nmol) to a final concentration of 1 mM. Assay mixtures were incubated for 10 minutes at 29ºC and stopped by addition of 0.5 ml of ice-cold 1 M perchloric acid. The amount of dCDP formed was determined by the standard method as described in (Thelander *et al.*, 1978). One unit of enzyme activity corresponds to 1 nmol of dCDP formed per minute of incubation. Km, Vmax and KL values were obtained by direct curve fitting as described (Climent *et al.*, 1992; Larsson Birgander *et al.*, 2005) by using the Kaleida-Graph software (Synergy Software).

7.4.8 - UV-vis absorption spectroscopy and decay of the tyrosyl radical site

Scanning spectra were registered on a Perkin Elmer Lambda2 spectrophotometer at 25° C, 20 µM monomer in 50 mM Tris-HCl, pH 7.6 were used. Radical decay of the *nrdF* protein was studied after addition of hydroxyurea (HU), final concentration ranging from 3.5 to 60 mM. Spectra were typically recorded between 300-750 nm in cycles of 150s with a scanning rate of 240 nm/min. End point spectrum, when radical absorption at 407 nm had decayed, was subtracted from start spectrum to give radical concentration using the extinction coefficient 3.25 $mM^{-1}cm^{-1}$ (Petersson *et al.*, 1980). Alternatively radical concentration was determined using the dropline procedure between 402 and 417 nm using e= 2.11 $mM^{-1}cm^{-1}$ in the equation $[A_{407}-(A_{402}-(5/15)*(A_{402}-A_{417}))]/e$. Kinetic traces at 407 nm were also run to determine the decay rate, incubations of *E. coli* with HU were run, at 410 nm, as controls. Spectra were run before and after the kinetic trace to measure degree of completeness of the reaction after subtractions as described above.

Direct reconstitution in the cuvette of the NrdF* protein was performed by addition of 1-4 µl of 5 mM Fe^{2+} in 0.1 M HCl to 500 µl protein solution giving 1-4 Fe/NrdF* polypeptide. Spectra were registered before addition of Fe^{2+} and the formation and decay of absorption bands were observed between 300-750 nm in cycles of 150 s with a scanning rate of 240 nm/min.

7.4.9 - EPR Spectroscopy

EPR measurements at Xband were performed either at ≥90 K on a Bruker ESP300 spectrometer using a nitrogen flow system or on a Bruker ESP500, for temperatures below 60 K, using a Oxford helium flow cryostat. Radical concentrations were determined, at unsaturated conditions, using a *Pseudomonas aeruginosa* NrdB sample and an *E. coli* NrdB sample with known tyrosyl radical concentrations as secondary standards. The error limits for radical quantitations are ≤±10%. Reconstituted samples for EPR measurements were either frozen with liquid nitrogen 6 minutes after addition of 2 Fe/polypeptide as described above or frozen after addition of 4 Fe/monomer followed by desalting on a NAP-5 column to remove unbound iron.

7.4.10 - Heterologous complementation assay

Class Ib *nrd* genes were amplified by PCR using primers pairs containing an RBS sequence from *E. coli* and SacI (upper primers) and PstI (lower primers) restriction sites (Table XI). The resulting fragments were purified and digested with SacI and PstI and inserted into pBAD18Km or pBAD33Cm (Guzman *et al.*, 1995) to generate the final constructs. p18-FI*Spyo, p18-IE*Spyo and p18-FE*Spyo were derived from p18-FIE*Spyo by enzymatic digestion (Table X).

In the heterologous complementation assay, constructs were transformed into the *E. coli* strain IG101 (and grown overnight at 30ºC in LB medium containing 50 µg/ml thymine plus the corresponding antibiotics. The following day, serial dilutions of the liquid cultures were plated onto LB agar plates containing the corresponding antibiotics plus 50 µg/ml thymine and either 0.3 % L-arabinose or 0.3 % D-glucose and were incubated at both 30 and 42ºC for 24-48 hours.

Plating efficiencies were determined as the fraction of CFU/ml under restrictive conditions compared to the number of CFU/ml under non-restrictive conditions.

7.4.11 - Image and data analysis

Images of each gel were captured with a Gel Doc 2000 station and the Quantity One Software package (Bio-Rad Laboratories).

7.5 - Part II Specific Procedures

7.5.1 - Bacterial strains, plasmids and growth media

The genotypes of the *E. coli* K-12 strains, plasmids and bacteriophages used are listed in Table IX.

Cultures were grown routinely at 37ºC with vigorous shaking in LB broth or on solid medium. When required, antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 34 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 17 μg/ml; rifampycine, 75 μg/ml; and toluidine blue, 260 μg/ml.

All DNA manipulations were performed as described in (Sambrook *et al.*, 1989).

7.5.2 - Site-directed Mutagenesis

A PCR fragment of 479 bp spanning over the 5' end of the *nrdD* gene (184 bp) and the upstream regulatory regions (295 bp) was amplified from *E. coli* MC1061 chromosomal DNA and ligated into the A/T cloning pGEM®-T easy vector (Promega), generating plasmid pIG72 (Table X). A two-step nested PCR mutagenesis was used over this plasmid to introduce the desired substitutions within the putative FNR boxes, using outside primers 45 and 46 (Table XI) and inside complementary primers (containing the modified FNR sequences) 47 and 48 and 49 and 50 to generate plasmids pIG73 (FNR-2d) and pIG74 (FNR-1d) (Table X).

7.5.3 - Construction of *nrdDG-lacZ* transcriptional fusions and mutant strains

In order to construct all the *nrdDG::lacZ* fusions, DNA fragments of 479 bp containing the 5' end of the *nrdD* gene and the upstream regulatory regions were digested from plasmids pIG72, pIG73 and pIG74 respectively, and ligated into the EcoRI and BamHI sites of plasmid pUJ8 (de Lorenzo *et al.*, 1990) to give transcriptional fusions between these fragments and the *lacZ* gene (Table X). All constructs were confirmed by PCR and DNA sequencing. Fusions were transferred to plasmid pUTminiTn5Km-2 (de Lorenzo *et al.*, 1990) by NotI digestion and introduced into the *E. coli* conjugative strain S-17 λ-pir. Biparental conjugation between S17-1 *λpir* and MC1061 Rif^R was used to force transposition and insertion of the fusions within the MC1061 chromosome, generating strains IG40, IG41 and IG42 (Table IX).

The *fnr* and *arcA* mutant strains were constructed by introducing the indicated mutation into strain IG40 by P1 transduction (Silhavy & Berman, 1984) followed by selection for the appropriate drug resistance (table IX). All *fnr* transductants were tested and proved not capable of growing in Minimal Media using glycerol and nitrates as the sole source of carbon and reducing power, respectively, indicative of the Fnr^- phenotype (Lambden & Guest, 1976). In a similar way, all *arcA* transductants were tested for sensitivity to toluidine blue dye (Roeder & Somerville, 1979).

7.5.4 - β-galactosidase assay

β-Galactosidase activities expressed from *nrdDG-lacZ* fusions were assayed according to Miller (Miller, 1972) using cultures grown in LB-broth plus glucose (0.5 %), either aerobically or anaerobically (in screw-cap tubes filled to the neck containing 3.2 mM sodium sulphide (Garriga *et al.*, 1996). The quoted specific activities (Miller units) are averages from duplicate samples of at least three independent cultures, variations being no more than 15 % from the mean.

7.5.5 - Purification of the FNR* protein

FNR*DA154 was overproduced from plasmid pGS771 (Table X), transformed into the *E. coli* BL21 DE3 strain, and purified from sonicated extracts of aerobic cultures grown in LB plus ampicillin (50 μg/ml), and harvested 3 to 4 hours after induction with 0.5 mM IPTG during mid-exponential phase. FNR* purification was performed on a 5 ml GSTrap FF column according to the manufacturer's general procedure (Pharmacia Biotech). FNR* was further purified and concentrated by ultrafiltration on a YM-10 Centriplus® (Millipore) equilibrated with Tris-HCl pH 7.5 and 1 mM DTT. Protein concentration was estimated by Bradford assay (Bradford, 1976) using the Coomassie® Protein Assay Reagent (Pierce), and purity was assessed on a 10 % SDS-polyacrylamide gel using a 30:0.8 acrylamide/bisacrylamide ratio (w/v).

7.5.6 - Electrophoretic Mobility Shift Assay (EMSA)

The FNR* binding to *nrdDG* was assessed by gel retardation assay as described by (Lazazzera *et al.*, 1993). Briefly, protein was mixed in a 10 μl volume with 0.5 μg of a 5'-end DIG labelled 479 bp DNA fragment containing the FNR-consensus sites to achieve a final concentration of: 0.1 M potassium glutamate (pH 7.5), 10 mM potassium phosphate (pH 7.5), 3 mM Tris-HCl (pH 7.9), 1 mM DTT, 50 μg/ml bovine serum albumin (Roche Diagnostics), and 5 % glycerol. Following incubation at 37 ºC for 10 minutes, the reaction mixture was loaded onto a 5 % polyacrylamide gel (30:0.8 Acrylamide:Bisacrylamide w/v) in 0.5x Tris Borate-EDTA buffer (Sambrook *et al.*, 1989), which had been electrophoresed for 30 minutes at 4 ºC prior to loading, and was then run at 110 V for two additional hours.

DNA was electrotransferred into a positively charged Nylon membrane (Roche Diagnostics) and developed by both chemiluminescence and colorimetric procedures according to the manufacturer's protocol (Roche Diagnostics).

8. - References

Aberg, A., Hahne, S., Karlsson, M., Larsson, A., Ormo, M., Ahgren, A. & Sjoberg, B. M. (1989). Evidence for two different classes of redox-active cysteines in ribonucleotide reductase of *Escherichia coli*. *J Biol Chem* **264**, 12249-12252.

Andersson, M. E., Hogbom, M., Rinaldo-Matthis, A., Blodig, W., Liang, Y., Persson, B. O., Sjoberg, B. M., Su, X. D. & Nordlund, P. (2004). Structural and mutational studies of the carboxylate cluster in iron-free ribonucleotide reductase R2. *Biochemistry* **43**, 7966-7972.

Aravind, L., Wolf, Y. I. & Koonin, E. V. (2000). The ATP-cone: an evolutionarily mobile, ATP-binding regulatory domain. *J Mol Microbiol Biotechnol* **2**, 191-194.

Aslund, F., Ehn, B., Miranda-Vizuete, A., Pueyo, C. & Holmgren, A. (1994). Two additional glutaredoxins exist in *Escherichia coli*: glutaredoxin 3 is a hydrogen donor for ribonucleotide reductase in a thioredoxin/glutaredoxin 1 double mutant. *Proc Natl Acad Sci U S A* **91**, 9813-9817.

Aslund, F., Berndt, K. D. & Holmgren, A. (1997). Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein-protein redox equilibria. *J Biol Chem* **272**, 30780-30786.

Aslund, F. & Beckwith, J. (1999). The thioredoxin superfamily: redundancy, specificity, and gray-area genomics. *J Bacteriol* **181**, 1375-1379.

Barcelona-Andres, B., Marina, A. & Rubio, V. (2002). Gene structure, organization, expression, and potential regulatory mechanisms of arginine catabolism in *Enterococcus faecalis*. *J Bacteriol* **184**, 6289-6300.

Barlow, T., Eliasson, R., Platz, A., Reichard, P. & Sjoberg, B. M. (1983). Enzymic modification of a tyrosine residue to a stable free radical in ribonucleotide reductase. *Proc Natl Acad Sci U S A* **80**, 1492-1495.

Becker, S., Holighaus, G., Gabrielczyk, T. & Unden, G. (1996). O_2 as the regulatory signal for FNR-dependent gene regulation in *Escherichia coli*. *J Bacteriol* **178**, 4515-4521.

Bentley, S. D., Maiwald, M., Murphy, L. D. & other authors (2003). Sequencing and analysis of the genome of the Whipple's disease bacterium *Tropheryma whipplei*. *Lancet* **361**, 637-644.

Beres, S. B., Sylva, G. L., Barbian, K. D. & other authors (2002). Genome sequence of a serotype M3 strain of group A *Streptococcus*: phage- encoded toxins, the high-virulence phenotype, and clone emergence. *Proc Natl Acad Sci U S A* **99**, 10078-10083.

Bianchi, V., Reichard, P., Eliasson, R., Pontis, E., Krook, M., Jornvall, H. & Haggard-Ljungquist, E. (1993). *Escherichia coli* ferredoxin NADP+ reductase: activation of *E. coli* anaerobic ribonucleotide reduction, cloning of the gene (*fpr*), and overexpression of the protein. *J Bacteriol* **175**, 1590-1595.

Bollinger, J. M., Jr., Tong, W. H., Ravi, N., Huynh, B. H., Edmondson, D. E. & Stubbe, J. A. (1995). Use of rapid kinetics methods to study the assembly of the diferric- tyrosyl radical cofactor of *E. coli* ribonucleotide reductase. *Methods Enzymol* **258**, 278-303.

Booker, S., Licht, S., Broderick, J. & Stubbe, J. (1994). Coenzyme B12-dependent ribonucleotide reductase: evidence for the participation of five cysteine residues in ribonucleotide reduction. *Biochemistry* **33**, 12676-12685.

Borovok, I., Kreisberg-Zakarin, R., Yanko, M., Schreiber, R., Myslovati, M., Aslund, F., Holmgren, A., Cohen, G. & Aharonowitz, Y. (2002). *Streptomyces* spp. contain class Ia and class II ribonucleotide reductases: expression analysis of the genes in vegetative growth. *Microbiology* **148**, 391-404.

Borovok, I., Gorovitz, B., Yanku, M., Schreiber, R., Gust, B., Chater, K., Aharonowitz, Y. & Cohen, G. (2004). Alternative oxygen-dependent and oxygen-independent ribonucleotide reductases in *Streptomyces*: cross-regulation and physiological role in response to oxygen limitation. *Mol Microbiol* **54**, 1022-1035.

Borovok, I., Gorovitz, B., Schreiber, R., Aharonowitz, Y. & Cohen, G. (2006). Coenzyme B12 controls transcription of the *Streptomyces* class Ia ribonucleotide reductase *nrdABS* operon via a riboswitch mechanism. *J Bacteriol* **188**, 2512-2520.

Boston, T. & Atlung, T. (2003). FNR-mediated oxygen-responsive regulation of the *nrdDG* operon of *Escherichia coli*. *J Bacteriol* **185**, 5310-5313.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.

Cerqueira, N. M., Pereira, S., Fernandes, P. A. & Ramos, M. J. (2005). Overview of ribonucleotide reductase inhibitors: an appealing target in anti-tumour therapy. *Curr Med Chem* **12**, 1283-1294.

Chabes, A., Domkin, V. & Thelander, L. (1999). Yeast Sml1, a protein inhibitor of ribonucleotide reductase. *J Biol Chem* **274**, 36679-36683.

Chabes, A., Domkin, V., Larsson, G., Liu, A., Graslund, A., Wijmenga, S. & Thelander, L. (2000). Yeast ribonucleotide reductase has a heterodimeric iron-radical-containing subunit. *Proc Natl Acad Sci U S A* **97**, 2474-2479.

Chabes, A. L., Pfleger, C. M., Kirschner, M. W. & Thelander, L. (2003). Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. *Proc Natl Acad Sci U S A* **100**, 3925-3929.

Chambaud, I., Heilig, R., Ferris, S. & other authors (2001). The complete genome sequence of the murine respiratory pathogen *Mycoplasma pulmonis*. *Nucleic Acids Res* **29**, 2145-2153.

Climent, I., Sjoberg, B. M. & Huang, C. Y. (1992). Site-directed mutagenesis and deletion of the carboxyl terminus of *Escherichia coli* ribonucleotide reductase protein R2. Effects on catalytic activity and subunit interaction. *Biochemistry* **31**, 4801-4807.

Cooperman, B. S. & Kashlan, O. B. (2003). A comprehensive model for the allosteric regulation of Class Ia ribonucleotide reductases. *Adv Enzyme Regul* **43**, 167-182.

Dawes, S. S., Warner, D. F., Tsenova, L., Timm, J., McKinney, J. D., Kaplan, G., Rubin, H. & Mizrahi, V. (2003). Ribonucleotide reduction in *Mycobacterium tuberculosis*: function and expression of genes encoding class Ib and class II ribonucleotide reductases. *Infect Immun* **71**, 6124-6131.

de Lorenzo, V., Herrero, M., Jakubzik, U. & Timmis, K. N. (1990). Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J Bacteriol* **172**, 6568-6572.

Drapal, N. & Sawers, G. (1995). Purification of ArcA and analysis of its specific interaction with the *pfl* promoter-regulatory region. *Mol Microbiol* **16**, 597-607.

Ekberg, M., Sahlin, M., Eriksson, M. & Sjoberg, B. M. (1996). Two conserved tyrosine residues in protein R1 participate in an intermolecular electron transfer in ribonucleotide reductase. *J Biol Chem* **271**, 20655-20659.

Ekberg, M., Potsch, S., Sandin, E., Thunnissen, M., Nordlund, P., Sahlin, M. & Sjoberg, B. M. (1998). Preserved catalytic activity in an engineered ribonucleotide reductase R2 protein with a nonphysiological radical transfer pathway. The importance of hydrogen bond connections between the participating residues. *J Biol Chem* **273**, 21003-21008.

Eklund, H., Uhlin, U., Farnegardh, M., Logan, D. T. & Nordlund, P. (2001). Structure and function of the radical enzyme ribonucleotide reductase. *Prog Biophys Mol Biol* **77**, 177-268.

Elgren, T. E., Lynch, J. B., Juarez-Garcia, C., Munck, E., Sjoberg, B. M. & Que, L., Jr. (1991). Electron transfer associated with oxygen activation in the B2 protein of ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **266**, 19265-19268.

Eliasson, R., Jornvall, H. & Reichard, P. (1986). Superoxide dismutase participates in the enzymatic formation of the tyrosine radical of ribonucleotide reductase from *Escherichia coli*. *Proc Natl Acad Sci U S A* **83**, 2373-2377.

Eliasson, R., Fontecave, M., Jornvall, H., Krook, M., Pontis, E. & Reichard, P. (1990). The anaerobic ribonucleoside triphosphate reductase from *Escherichia coli* requires S-adenosylmethionine as a cofactor. *Proc Natl Acad Sci U S A* **87**, 3314-3318.

Eliasson, R., Pontis, E., Eckstein, F. & Reichard, P. (1994a). Interactions of 2'-modified azido- and haloanalogs of deoxycytidine 5'- triphosphate with the anaerobic ribonucleotide reductase of *Escherichia coli*. *J Biol Chem* **269**, 26116-26120.

Eliasson, R., Pontis, E., Sun, X. & Reichard, P. (1994b). Allosteric control of the substrate specificity of the anaerobic ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **269**, 26052-26057.

Eliasson, R., Pontis, E., Jordan, A. & Reichard, P. (1996). Allosteric regulation of the third ribonucleotide reductase (NrdEF enzyme) from enterobacteriaceae. *J Biol Chem* **271**, 26582-26587.

Eliasson, R., Pontis, E., Jordan, A. & Reichard, P. (1999). Allosteric control of three B12-dependent (class II) ribonucleotide reductases. Implications for the evolution of ribonucleotide reduction. *J Biol Chem* **274**, 7182-7189.

Elledge, S. J. & Davis, R. W. (1989). Identification of the DNA damage-responsive element of RNR2 and evidence that four distinct cellular factors bind it. *Mol Cell Biol* **9**, 5373-5386.

Elledge, S. J. & Davis, R. W. (1990). Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. *Genes Dev* **4**, 740-751.

Engel, P., Trageser, M. & Unden, G. (1991). Reversible interconversion of the functional state of the gene regulator FNR from *Escherichia coli* in vivo by O_2 and iron availability. *Arch Microbiol* **156**, 463-470.

Engstrom, Y., Eriksson, S., Jildevik, I., Skog, S., Thelander, L. & Tribukait, B. (1985). Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits. *J Biol Chem* **260**, 9114-9116.

Eriksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnstrom, K., Sjoberg, B. M. & Eklund, H. (1997). Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. *Structure* **5**, 1077-1092.

Eriksson, M., Jordan, A. & Eklund, H. (1998). Structure of *Salmonella typhimurium nrdF* ribonucleotide reductase in its oxidized and reduced forms. *Biochemistry* **37**, 13359-13369.

Federle, M. J., McIver, K. S. & Scott, J. R. (1999). A response regulator that represses transcription of several virulence operons in the group A *streptococcus*. *J Bacteriol* **181**, 3649-3657.

Ferretti, J. J., McShan, W. M., Ajdic, D. & other authors (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A* **98**, 4658-4663.

Fieschi, F., Torrents, E., Toulokhonova, L., Jordan, A., Hellman, U., Barbe, J., Gibert, I., Karlsson, M. & Sjoberg, B. M. (1998). The manganese-containing ribonucleotide reductase of *Corynebacterium ammoniagenes* is a class Ib enzyme. *J Biol Chem* **273**, 4329-4337.

Filatov, D. & Thelander, L. (1995). Role of a proximal NF-Y binding promoter element in S phase-specific expression of mouse ribonucleotide reductase R2 gene. *J Biol Chem* **270**, 25239-25243.

Filatov, D., Bjorklund, S., Johansson, E. & Thelander, L. (1996). Induction of the mouse ribonucleotide reductase R1 and R2 genes in response to DNA damage by UV light. *J Biol Chem* **271**, 23698-23704.

Filpula, D. & Fuchs, J. A. (1977). Regulation of ribonucleoside diphosphate reductase synthesis in *Escherichia coli*: increased enzyme synthesis as a result of inhibition of deoxyribonucleic acid synthesis. *J Bacteriol* **130**, 107-113.

Fink, R. C., Evans, M. R., Porwollik, S., Vazquez-Torres, A., Jones-Carson, J., Troxell, B., Libby, S. J., McClelland, M. & Hassan, H. M. (2007). FNR Is a Global Regulator of Virulence and Anaerobic Metabolism in *Salmonella enterica Serovar Typhimurium* (ATCC 14028s). *J Bacteriol* **189**, 2262-2273.

Fontecave, M., Eliasson, R. & Reichard, P. (1987). NAD(P)H:flavin oxidoreductase of *Escherichia coli*. A ferric iron reductase participating in the generation of the free radical of ribonucleotide reductase. *J Biol Chem* **262**, 12325-12331.

Fontecave, M., Eliasson, R. & Reichard, P. (1989). Enzymatic regulation of the radical content of the small subunit of *Escherichia coli* ribonucleotide reductase involving reduction of its redox centers. *J Biol Chem* **264**, 9164-9170.

Fontecave, M., Nordlund, P., Eklund, H. & Reichard, P. (1992). The redox centers of ribonucleotide reductase of *Escherichia coli*. *Adv Enzymol Relat Areas Mol Biol* **65**, 147-183.

Fontecave, M., Mulliez, E. & Logan, D. T. (2002). Deoxyribonucleotide synthesis in anaerobic microorganisms: the class III ribonucleotide reductase. *Prog Nucleic Acid Res Mol Biol* **72**, 95-127.

Fraser, C. M., Gocayne, J. D., White, O. & other authors (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**, 397-403.

Fu, H. A., Iuchi, S. & Lin, E. C. (1991). The requirement of ArcA and Fnr for peak expression of the *cyd* operon in *Escherichia coli* under microaerobic conditions. *Mol Gen Genet* **226**, 209-213.

Gambarelli, S., Luttringer, F., Padovani, D., Mulliez, E. & Fontecave, M. (2005). Activation of the anaerobic ribonucleotide reductase by s-adenosylmethionine. *Chembiochem* **6**, 1960-1962.

Garriga, X., Eliasson, R., Torrents, E., Jordan, A., Barbe, J., Gibert, I. & Reichard, P. (1996). *nrdD* and *nrdG* genes are essential for strict anaerobic growth of *Escherichia coli*. *Biochem Biophys Res Commun* **229**, 189-192.

Ge, J., Perlstein, D. L., Nguyen, H. H., Bar, G., Griffin, R. G. & Stubbe, J. (2001). Why multiple small subunits (Y2 and Y4) for yeast ribonucleotide reductase? Toward understanding the role of Y4. *Proc Natl Acad Sci U S A* **98**, 10067-10072.

Gerfen, G. J., Licht, S., Willems, J. P., Hoffman, B. M. & Stubbe, J. (1996). Electron paramagnetic resonance investigations of a kinetically competent intermediate formed in ribonucleotide reduction: Evidence for a thiyl radical-Cob(II)alamin interaction. *J Am Chem Soc* **118**, 8192-8197.

Gilbert, W. (1986). Origin of life: The RNA world. *Nature* **319**, 618.

Gleason, F. K. & Holmgren, A. (1988). Thioredoxin and related proteins in procaryotes. *FEMS Microbiol Rev* **4**, 271-297.

Gon, S., Faulkner, M. J. & Beckwith, J. (2006). In vivo requirement for glutaredoxins and thioredoxins in the reduction of the ribonucleotide reductases of *Escherichia coli*. *Antioxid Redox Signal* **8**, 735-742.

Green, J., Scott, C. & Guest, J. R. (2001). Functional versatility in the CRP-FNR superfamily of transcription factors: FNR and FLP. *Adv Microb Physiol* **44**, 1-34.

Grinberg, I., Shteinberg, T., Gorovitz, B., Aharonowitz, Y., Cohen, G. & Borovok, I. (2006). The *Streptomyces* NrdR transcriptional regulator is a Zn ribbon/ATP cone protein that binds to the promoter regions of class Ia and class II ribonucleotide reductase operons. *J Bacteriol* **188**, 7635-7644.

Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177**, 4121-4130.

Han, J. S., Kwon, H. S., Yim, J. B. & Hwang, D. S. (1998). Effect of IciA protein on the expression of the *nrd* gene encoding ribonucleoside diphosphate reductase in *E. coli*. *Mol Gen Genet* **259**, 610-614.

Hanke, P. D. & Fuchs, J. A. (1983a). Characterization of the mRNA coding for ribonucleoside diphosphate reductase in *Escherichia coli*. *J Bacteriol* **156**, 1192-1197.

Hanke, P. D. & Fuchs, J. A. (1983b). Regulation of ribonucleoside diphosphate reductase mRNA synthesis in *Escherichia coli*. *J Bacteriol* **154**, 1040-1045.

Hantke, K. (2001). Iron and metal regulation in bacteria. *Curr Opin Microbiol* **4**, 172-177.

Higgins, D., Thompson, J., Gibson, T. T. J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressivemultiple sequence alignment through sequence weighting,position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Himmelreich, R., Hilbert, H., Plagens, H., Pirkl, E., Li, B. C. & Herrmann, R. (1996). Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. *Nucleic Acids Res* **24**, 4420-4449.

Hogbom, M., Huque, Y., Sjoberg, B. M. & Nordlund, P. (2002). Crystal structure of the di-iron/radical protein of ribonucleotide reductase from *Corynebacterium ammoniagenes*. *Biochemistry* **41**, 1381-1389.

Hogbom, M., Stenmark, P., Voevodskaya, N., McClarty, G., Graslund, A. & Nordlund, P. (2004). The radical site in chlamydial ribonucleotide reductase defines a new R2 subclass. *Science* **305**, 245-248.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J Biol Chem* **264**, 13963-13966.

Holmgren, A. & Aslund, F. (1995). Glutaredoxin. *Methods Enzymol* **252**, 283-292.

Holmgren, A. & Bjornstedt, M. (1995). Thioredoxin and thioredoxin reductase. *Methods Enzymol* **252**, 199-208.

Huque, Y., Fieschi, F., Torrents, E., Gibert, I., Eliasson, R., Reichard, P., Sahlin, M. & Sjoberg, B. M. (2000). The active form of the R2F protein of class Ib ribonucleotide reductase from *Corynebacterium ammoniagenes* is a diferric protein. *J Biol Chem* **275**, 25365-25371.

Husmann, L. K., Yung, D. L., Hollingshead, S. K. & Scott, J. R. (1997). Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia. *Infect Immun* **65**, 1422-1430.

Iuchi, S., Aristarkhov, A., Dong, J. M., Taylor, J. S. & Lin, E. C. (1994). Effects of nitrate respiration on expression of the Arc-controlled operons encoding succinate dehydrogenase and flavin-linked L-lactate dehydrogenase. *J Bacteriol* **176**, 1695-1701.

Jacobson, B. A. & Fuchs, J. A. (1998). Multiple cis-acting sites positively regulate *Escherichia coli nrd* expression. *Mol Microbiol* **28**, 1315-1322.

Johansson, E., Skogman, E. & Thelander, L. (1995). The TATA-less promoter of mouse ribonucleotide reductase R1 gene contains a TFII-I binding initiator element essential for cell cycle-regulated transcription. *J Biol Chem* **270**, 30162-30167.

Jordan, A., Gibert, I. & Barbe, J. (1994a). Cloning and sequencing of the genes from *Salmonella typhimurium* encoding a new bacterial ribonucleotide reductase. *J Bacteriol* **176**, 3420-3427.

Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbe, J. & Reichard, P. (1994b). A second class I ribonucleotide reductase in *Enterobacteriaceae*: characterization of the *Salmonella typhimurium* enzyme. *Proc Natl Acad Sci U S A* **91**, 12892-12896.

Jordan, A., Aragall, E., Gibert, I. & Barbe, J. (1996a). Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli nrdEF* operons encoding one of two class I ribonucleotide reductases present in both bacteria. *Mol Microbiol* **19**, 777-790.

Jordan, A., Pontis, E., Aslund, F., Hellman, U., Gibert, I. & Reichard, P. (1996b). The ribonucleotide reductase system of *Lactococcus lactis*. Characterization of an NrdEF enzyme and a new electron transport protein.
J Biol Chem **271**, 8779-8785.

Jordan, A., Aslund, F., Pontis, E., Reichard, P. & Holmgren, A. (1997). Characterization of *Escherichia coli* NrdH. A glutaredoxin-like protein with a thioredoxin-like activity profile. *J Biol Chem* **272**, 18044-18050.

Jordan, A. & Reichard, P. (1998). Ribonucleotide reductases. *Annu Rev Biochem* **67**, 71-98.

Jordan, A., Torrents, E., Sala, I., Hellman, U., Gibert, I. & Reichard, P. (1999). Ribonucleotide reduction in *Pseudomonas* species: simultaneous presence of active enzymes from different classes. *J Bacteriol* **181**, 3974-3980.

Kalman, S., Mitchell, W., Marathe, R. & other authors (1999). Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. *Nat Genet* **21**, 385-389.

Kashlan, O. B. & Cooperman, B. S. (2003). Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. *Biochemistry* **42**, 1696-1706.

Kasrayan, A., Birgander, P. L., Pappalardo, L., Regnstrom, K., Westman, M., Slaby, A., Gordon, E. & Sjoberg, B. M. (2004). Enhancement by effectors and substrate nucleotides of R1-R2 interactions in *Escherichia coli* class Ia ribonucleotide reductase. *J Biol Chem* **279**, 31050-31057.

Kauppi, B., Nielsen, B. B., Ramaswamy, S., Larsen, I. K., Thelander, M., Thelander, L. & Eklund, H. (1996). The three-dimensional structure of mammalian ribonucleotide reductase protein R2 reveals a more-accessible iron-radical site than *Escherichia coli* R2. *J Mol Biol* **262**, 706-720.

Kiley, P. J. & Reznikoff, W. S. (1991). Fnr mutants that activate gene expression in the presence of oxygen. *J Bacteriol* **173**, 16-22.

Kiley, P. J. & Beinert, H. (1998). Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol Rev* **22**, 341-352.

King, D. S. & Reichard, P. (1995). Mass spectrometric determination of the radical scission site in the anaerobic ribonucleotide reductase of *Escherichia coli*. *Biochem Biophys Res Commun* **206**, 731-735.

Knappe, J. & Wagner, A. F. (1995). Glycyl free radical in pyruvate formate-lyase: synthesis, structure characteristics, and involvement in catalysis. *Methods Enzymol* **258**, 343-362.

Kobayashi, K., Ehrlich, S. D., Albertini, A. & other authors (2003). Essential *Bacillus subtilis* genes. *Proc Natl Acad Sci U S A* **100**, 4678-4683.

Koonin, E. V. (2000). How many genes can make a cell: the minimal-gene-set concept. *Annu Rev Genomics Hum Genet* **1**, 99-116.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* **5**, 150-163.

Kunz, B. A., Kohalmi, S. E., Kunkel, T. A., Mathews, C. K., McIntosh, E. M. & Reidy, J. A. (1994). International Commission for Protection Against Environmental Mutagens and Carcinogens. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutat Res* **318**, 1-64.

Lambden, P. R. & Guest, J. R. (1976). Mutants of *Escherichia coli* K12 unable to use fumarate as an anaerobic electron acceptor. *J Gen Microbiol* **97**, 145-160.

Larralde, R., Robertson, M. P. & Miller, S. L. (1995). Rates of decomposition of ribose and other sugars: implications for chemical evolution. *Proc Natl Acad Sci U S A* **92**, 8158-8160.

Larsson, A. & Sjoberg, B. M. (1986). Identification of the stable free radical tyrosine residue in ribonucleotide reductase. *Embo J* **5**, 2037-2040.

Larsson Birgander, P., Bug, S., Kasrayan, A., Dahlroth, S. L., Westman, M., Gordon, E. & Sjoberg, B. M. (2005). Nucleotide-dependent formation of catalytically competent dimers from engineered monomeric ribonucleotide reductase protein R1. *J Biol Chem*.

Larsson, K. M., Andersson, J., Sjoberg, B. M., Nordlund, P. & Logan, D. T. (2001). Structural basis for allosteric substrate specificity regulation in anaerobic ribonucleotide reductases. *Structure (Camb)* **9**, 739-750.

Larsson, K. M., Jordan, A., Eliasson, R., Reichard, P., Logan, D. T. & Nordlund, P. (2004a). Structural mechanism of allosteric substrate specificity regulation in a ribonucleotide reductase. *Nat Struct Mol Biol* **11**, 1142-1149.

Larsson, P. L., Kasrayan, A. & Sjoberg, B. M. (2004b). Mutant R1 proteins from *Escherichia coli* class Ia ribonucleotide reductase with altered responses to dATP inhibition. *J Biol Chem*.

Lawrence, C. C. & Stubbe, J. (1998). The function of adenosylcobalamin in the mechanism of ribonucleoside triphosphate reductase from *Lactobacillus leichmannii*. *Curr Opin Chem Biol* **2**, 650-655.

Lazazzera, B. A., Bates, D. M. & Kiley, P. J. (1993). The activity of the *Escherichia coli* transcription factor FNR is regulated by a change in oligomeric state. *Genes Dev* **7**, 1993-2005.

Licht, S., Gerfen, G. J. & Stubbe, J. (1996). Thiyl radicals in ribonucleotide reductases. *Science* **271**, 477-481.

Licht, S., Lawrence, C. C. & Stubbe, J. (1999). Class II Ribonucleotide Reductases Catalyze Carbon-Cobalt Bond Reformation on Every Turnover. *J Am Chem Soc* **121**, 7463-7468.

Lin, Z. P., Belcourt, M. F., Cory, J. G. & Sartorelli, A. C. (2004). Stable suppression of the R2 subunit of ribonucleotide reductase by R2-targeted siRNA sensitizes p53(-/-) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors. *J Biol Chem*.

Liu, A., Potsch, S., Davydov, A., Barra, A. L., Rubin, H. & Graslund, A. (1998). The tyrosyl free radical of recombinant ribonucleotide reductase from *Mycobacterium tuberculosis* is located in a rigid hydrophobic pocket. *Biochemistry* **37**, 16369-16377.

Logan, D. T., Andersson, J., Sjoberg, B. M. & Nordlund, P. (1999). A glycyl radical site in the crystal structure of a class III ribonucleotide reductase. *Science* **283**, 1499-1504.

Logan, D. T., Mulliez, E., Larsson, K. M., Bodevin, S., Atta, M., Garnaud, P. E., Sjoberg, B. M. & Fontecave, M. (2003). A metal-binding site in the catalytic subunit of anaerobic ribonucleotide reductase. *Proc Natl Acad Sci U S A* **100**, 3826-3831.

Ludwig, M. L. & Matthews, R. G. (2002). Effector regulation in a monomeric enzyme. *Nat Struct Biol* **9**, 236-238.

Lynch, A. S. & Lin, E. C. (1996a). Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. *J Bacteriol* **178**, 6238-6249.

Lynch, A. S. & Lin, E. C. C. (1996b). Responses to molecular oxygen, p.1526-1538. *In F C Neidhardt, R Curtiss III, J L Ingraham, E C C Lin, K B Low, Jr, B Magasanik, W S Reznikoff, M Schaechter, and H E Umbarger (ed), Escherichia coli and Salmonella: cellular and molecular biology, 2nd ed American Society for Microbiology, Washington, D C*.

Mann, G. J., Graslund, A., Ochiai, E., Ingemarson, R. & Thelander, L. (1991). Purification and characterization of recombinant mouse and herpes simplex virus ribonucleotide reductase R2 subunit. *Biochemistry* **30**, 1939-1947.

Mao, S. S., Holler, T. P., Yu, G. X., Bollinger, J. M., Jr., Booker, S., Johnston, M. I. & Stubbe, J. (1992). A model for the role of multiple cysteine residues involved in ribonucleotide reduction: amazing and still confusing. *Biochemistry* **31**, 9733-9743.

Martin, J. L. (1995). Thioredoxin--a fold for all reasons. *Structure* **3**, 245-250.

McLaughlin, R. E. & Ferretti, J. J. (1995). Electrotransformation of *Streptococci*. *Methods Mol Biol* **47**, 185-193.

Meng, W., Green, J. & Guest, J. R. (1997). FNR-dependent repression of *ndh* gene expression requires two upstream FNR-binding sites. *Microbiology* **143**, 1521-1532.

Miller, J. M. (1972). Experiments in Molecular Genetics. *Cold Spring Harbor, NY: Cold Spring Harbor Laboratory*.

Miranda-Vizuete, A., Damdimopoulos, A. E., Gustafsson, J. & Spyrou, G. (1997). Cloning, expression, and characterization of a novel *Escherichia coli* thioredoxin. *J Biol Chem* **272**, 30841-30847.

Monje-Casas, F., Jurado, J., Prieto-Alamo, M. J., Holmgren, A. & Pueyo, C. (2001). Expression analysis of the *nrdHIEF* operon from *Escherichia coli*. Conditions that trigger the transcript level in vivo. *J Biol Chem* **276**, 18031-18037.

Mulliez, E., Ollagnier, S., Fontecave, M., Eliasson, R. & Reichard, P. (1995). Formate is the hydrogen donor for the anaerobic ribonucleotide reductase from *Escherichia coli*. *Proc Natl Acad Sci U S A* **92**, 8759-8762.

Mulliez, E., Padovani, D., Atta, M., Alcouffe, C. & Fontecave, M. (2001). Activation of class III ribonucleotide reductase by flavodoxin: a protein radical-driven electron transfer to the iron-sulfur center. *Biochemistry* **40**, 3730-3736.

Nakano, K., Balint, E., Ashcroft, M. & Vousden, K. H. (2000). A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene* **19**, 4283-4289.

Nelson, D. L. & Cox, M. M. (2000). *Lehninger Principles of Biochemistry*, 3th. edn: Worth Publishers.

Ng, W. V., Kennedy, S. P., Mahairas, G. G. & other authors (2000). Genome sequence of *Halobacterium* species NRC-1. *Proc Natl Acad Sci U S A* **97**, 12176-12181.

Nicholas, K. B. & Nicholas, H. B. J. (1997). GeneDoc: a tool for editing and annotating multiple sequence alignments vs.2.6.001. *Distributed by the authors*.

Nordlund, P., Sjoberg, B. M. & Eklund, H. (1990). Three-dimensional structure of the free radical protein of ribonucleotide reductase. *Nature* **345**, 593-598.

Nordlund, P. & Eklund, H. (1993). Structure and function of the *Escherichia coli* ribonucleotide reductase protein R2. *J Mol Biol* **232**, 123-164.

Nordlund, P. & Reichard, P. (2006). Ribonucleotide reductases. *Annu Rev Biochem* **75**, 681-706.

Nyholm, S., Thelander, L. & Graslund, A. (1993). Reduction and loss of the iron center in the reaction of the small subunit of mouse ribonucleotide reductase with hydroxyurea. *Biochemistry* **32**, 11569-11574.

Ochiai, E., Mann, G. J., Graslund, A. & Thelander, L. (1990). Tyrosyl free radical formation in the small subunit of mouse ribonucleotide reductase. *J Biol Chem* **265**, 15758-15761.

Oehlmann, W. & Auling, G. (1999). Ribonucleotide reductase (RNR) of *Corynebacterium glutamicum* ATCC 13032--genetic characterization of a second class IV enzyme. *Microbiology* **145**, 1595-1604.

Ollagnier, S., Mulliez, E., Gaillard, J., Eliasson, R., Fontecave, M. & Reichard, P. (1996). The anaerobic *Escherichia coli* ribonucleotide reductase. Subunit structure and iron sulfur center. *J Biol Chem* **271**, 9410-9416.

Ollagnier, S., Mulliez, E., Schmidt, P. P. & other authors (1997). Activation of the anaerobic ribonucleotide reductase from *Escherichia coli*. The essential role of the iron-sulfur center for S- adenosylmethionine reduction. *J Biol Chem* **272**, 24216-24223.

Ortenberg, R., Gon, S., Porat, A. & Beckwith, J. (2004). Interactions of glutaredoxins, ribonucleotide reductase, and components of the DNA replication system of *Escherichia coli*. *Proc Natl Acad Sci U S A* **101**, 7439-7444.

Panagou, D., Orr, M. D., Dunstone, J. R. & Blakley, R. L. (1972). A monomeric, allosteric enzyme with a single polypeptide chain. Ribonucleotide reductase of *Lactobacillus leichmannii*. *Biochemistry* **11**, 2378-2388.

Park, S. J. & Gunsalus, R. P. (1995). Oxygen, iron, carbon, and superoxide control of the fumarase fumA and fumC genes of *Escherichia coli*: role of the arcA, fnr, and soxR gene products. *J Bacteriol* **177**, 6255-6262.

Perlstein, D. L., Ge, J., Ortigosa, A. D., Robblee, J. H., Zhang, Z., Huang, M. & Stubbe, J. (2005). The active form of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit is a heterodimer in vitro and in vivo. *Biochemistry* **44**, 15366-15377.

Petersson, L., Graslund, A., Ehrenberg, A., Sjoberg, B. M. & Reichard, P. (1980). The iron center in ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **255**, 6706-6712.

Platz, A. & Sjoberg, B. M. (1980). Construction and characterization of hybrid plasmids containing the *Escherichia coli nrd* region. *J Bacteriol* **143**, 561-568.

Poole, A. M., Logan, D. T. & Sjoberg, B. M. (2002). The evolution of the ribonucleotide reductases: much ado about oxygen. *J Mol Evol* **55**, 180-196.

Prinz, W. A., Aslund, F., Holmgren, A. & Beckwith, J. (1997). The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J Biol Chem* **272**, 15661-15667.

Reichard, P. & Rutberg, L. (1960). Formation of deoxycytidine 5'-phosphate from cytidine 5'-phosphate with enzymes from *Escherichia coli*. *Biochim Biophys Acta* **37**, 554-555.

Reichard, P. (1962). Enzymatic synthesis of deoxyribonucleotides. I. Formation of deoxycytidine diphosphate from cytidine diphosphate with enzymes from *Escherichia coli*. *J Biol Chem* **237**, 3513-3519.

Reichard, P. (1993). The anaerobic ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **268**, 8383-8386.

Reichard, P. (1997). The evolution of ribonucleotide reduction. *Trends Biochem Sci* **22**, 81-85.

Reichard, P. (2002). Ribonucleotide reductases: the evolution of allosteric regulation. *Arch Biochem Biophys* **397**, 149-155.

Ritz, D. & Beckwith, J. (2001). Roles of thiol-redox pathways in bacteria. *Annu Rev Microbiol* **55**, 21-48.

Rodionov, D. A. & Gelfand, M. S. (2005). Identification of a bacterial regulatory system for ribonucleotide reductases by phylogenetic profiling. *Trends Genet* **21**, 385-389.

Roeder, W. & Somerville, R. L. (1979). Cloning the trpR gene. *Mol Gen Genet* **176**, 361-368.

Rova, U., Goodtzova, K., Ingemarson, R., Behravan, G., Graslund, A. & Thelander, L. (1995). Evidence by site-directed mutagenesis supports long-range electron transfer in mouse ribonucleotide reductase. *Biochemistry* **34**, 4267-4275.

Rova, U., Adrait, A., Potsch, S., Graslund, A. & Thelander, L. (1999). Evidence by mutagenesis that Tyr(370) of the mouse ribonucleotide reductase R2 protein is the connecting link in the intersubunit radical transfer pathway. *J Biol Chem* **274**, 23746-23751.

Sahlin, M., Petersson, L., Graslund, A., Ehrenberg, A., Sjoberg, B. M. & Thelander, L. (1987). Magnetic interaction between the tyrosyl free radical and the antiferromagnetically coupled iron center in ribonucleotide reductase. *Biochemistry* **26**, 5541-5548.

Sahlin, M., Sjoberg, B. M., Backes, G., Loehr, T. & Sanders-Loehr, J. (1990). Activation of the iron-containing B2 protein of ribonucleotide reductase by hydrogen peroxide. *Biochem Biophys Res Commun* **167**, 813-818.

Saleh, L. & Bollinger, J. M., Jr. (2006). Cation mediation of radical transfer between Trp48 and Tyr356 during O_2 activation by protein R2 of *Escherichia coli* ribonucleotide reductase: relevance to R1-R2 radical transfer in nucleotide reduction? *Biochemistry* **45**, 8823-8830.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual 2nd ed. *Cold Spring Harbor Lab Press, Plainview, NY*.

Sancho, J. (2006). Flavodoxins: sequence, folding, binding, function and beyond. *Cell Mol Life Sci* **63**, 855-864.

Schmidt, P. P., Andersson, K. K., Barra, A. L., Thelander, L. & Graslund, A. (1996). High field EPR studies of mouse ribonucleotide reductase indicate hydrogen bonding of the tyrosyl radical. *J Biol Chem* **271**, 23615-23618.

Scotti, C., Valbuzzi, A., Perego, M., Galizzi, A. & Albertini, A. M. (1996). The *Bacillus subtilis* genes for ribonucleotide reductase are similar to the genes for the second class I NrdE/NrdF enzymes of *Enterobacteriaceae*. *Microbiology* **142**, 2995-3004.

Shaw, D. J. & Guest, J. R. (1982). Amplification and product identification of the *fnr* gene of *Escherichia coli*. *J Gen Microbiol* **128**, 2221-2228.

Shen, J. & Gunsalus, R. P. (1997). Role of multiple ArcA recognition sites in anaerobic regulation of succinate dehydrogenase (sdhCDAB) gene expression in *Escherichia coli*. *Mol Microbiol* **26**, 223-236.

Silhavy, T., M. & Berman, a. L. E. (1984). *Experiments with gene fusions*: Cold Spring Harbor, N.Y.

Sintchak, M. D., Arjara, G., Kellogg, B. A., Stubbe, J. & Drennan, C. L. (2002). The crystal structure of class II ribonucleotide reductase reveals how an allosterically regulated monomer mimics a dimer. *Nat Struct Biol* **9**, 293-300.

Sjoberg, B. M., Karlsson, M. & Jornvall, H. (1987). Half-site reactivity of the tyrosyl radical of ribonucleotide reductase from *Escherichia coli*. *J Biol Chem* **262**, 9736-9743.

Smalley, D., Rocha, E. R. & Smith, C. J. (2002). Aerobic-type ribonucleotide reductase in the anaerobe *Bacteroides fragilis*. *J Bacteriol* **184**, 895-903.

Smoot, J. C., Barbian, K. D., Van Gompel, J. J. & other authors (2002). Genome sequence and comparative microarray analysis of serotype M18 group A *Streptococcus* strains associated with acute rheumatic fever outbreaks. *Proc Natl Acad Sci U S A* **99**, 4668-4673.

Sofia, H. J., Chen, G., Hetzler, B. G., Reyes-Spindola, J. F. & Miller, N. E. (2001). Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* **29**, 1097-1106.

Stephens, R. S., Kalman, S., Lammel, C. & other authors (1998). Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**, 754-759.

Stewart, E. J., Aslund, F. & Beckwith, J. (1998). Disulfide bond formation in the *Escherichia coli* cytoplasm: an in vivo role reversal for the thioredoxins. *Embo J* **17**, 5543-5550.

Storz, G. & Imlay, J. A. (1999). Oxidative stress. *Curr Opin Microbiol* **2**, 188-194.

Strand, K. R., Karlsen, S., Kolberg, M., Rohr, A. K., Gorbitz, C. H. & Andersson, K. K. (2004). Crystal structural studies of changes in the native dinuclear iron center of ribonucleotide reductase protein R2 from mouse. *J Biol Chem* **279**, 46794-46801.

Stubbe, J. (1990). Ribonucleotide reductases. *Adv Enzymol Relat Areas Mol Biol* **63**, 349-419.

Stubbe, J., Ge, J. & Yee, C. S. (2001). The evolution of ribonucleotide reduction revisited. *Trends Biochem Sci* **26**, 93-99.

Stubbe, J., Nocera, D. G., Yee, C. S. & Chang, M. C. (2003). Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? *Chem Rev* **103**, 2167-2201.

Sun, L., Jacobson, B. A., Dien, B. S., Srienc, F. & Fuchs, J. A. (1994). Cell cycle regulation of the *Escherichia coli nrd* operon: requirement for a cis-acting upstream AT-rich sequence. *J Bacteriol* **176**, 2415-2426.

Sun, X., Eliasson, R., Pontis, E., Andersson, J., Buist, G., Sjoberg, B. M. & Reichard, P. (1995). Generation of the glycyl radical of the anaerobic *Escherichia coli* ribonucleotide reductase requires a specific activating enzyme. *J Biol Chem* **270**, 2443-2446.

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. & Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* **404**, 42-49.

Tao, H., Bausch, C., Richmond, C., Blattner, F. R. & Conway, T. (1999). Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media. *J Bacteriol* **181**, 6425-6440.

Tao, X., Schiering, N., Zeng, H. Y., Ringe, D. & Murphy, J. R. (1994). Iron, DtxR, and the regulation of diphtheria toxin expression. *Mol Microbiol* **14**, 191-197.

Tartoff, K. D. & Hobbs, C. A. (1987). Improved media for growing plasmid and cosmid clones. *Bethesda Res Lab Focus*, 9:12.

Thelander, L., Sjoberg, B. R. & Eriksson, S. (1978). Ribonucleoside diphosphate reductase (*Escherichia coli*). *Methods Enzymol* **51**, 227-237.

Thelander, L. & Graslund, A. (1993). Ribonucleotide reductase in mammalian systems. In *Metal Ions in Biologial Systems*, pp. 109-129. Edited by H. a. A. Sigel. New York: Marcel Dekker Inc.

Tong, W., Burdi, D., Riggs-Gelasco, P. & other authors (1998). Characterization of Y122F R2 of *Escherichia coli* ribonucleotide reductase by time-resolved physical biochemical methods and X-ray crystallography. *Biochemistry* **37**, 5840-5848.

Torrents, E., Eliasson, R., Wolpher, H., Graslund, A. & Reichard, P. (2001). The anaerobic ribonucleotide reductase from *Lactococcus lactis*. Interactions between the two proteins NrdD and NrdG. *J Biol Chem* **276**, 33488-33494.

Torrents, E., Aloy, P., Gibert, I. & Rodriguez-Trelles, F. (2002). Ribonucleotide reductases: divergent evolution of an ancient enzyme. *J Mol Evol* **55**, 138-152.

Torrents, E., Roca, I. & Gibert, I. (2003). *Corynebacterium ammoniagenes* class Ib ribonucleotide reductase: transcriptional regulation of an atypical genomic organization in the *nrd* cluster. *Microbiology* **149**, 1011-1020.

Torrents, E., Poplawski, A. & Sjoberg, B. M. (2005a). Two proteins mediate class II ribonucleotide reductase activity in *Pseudomonas aeruginosa*: expression and transcriptional analysis of the aerobic enzymes. *J Biol Chem* **280**, 16571-16578.

Torrents, E., Sahlin, M., Biglino, D., Graslund, A. & Sjoberg, B. M. (2005b). Efficient growth inhibition of *Bacillus anthracis* by knocking out the ribonucleotide reductase tyrosyl radical. *Proc Natl Acad Sci U S A*.

Torrents, E., Trevisiol, C., Rotte, C., Hellman, U., Martin, W. & Reichard, P. (2006). *Euglena gracilis* ribonucleotide reductase: the eukaryote class II enzyme and the possible antiquity of eukaryote B12 dependence. *J Biol Chem* **281**, 5604-5611.

Tuggle, C. K. & Fuchs, J. A. (1986). Regulation of the operon encoding ribonucleotide reductase in *Escherichia coli*: evidence for both positive and negative control. *Embo J* **5**, 1077-1085.

Tuggle, C. K. & Fuchs, J. A. (1990). Regulation of the operon encoding ribonucleotide reductase: role of the negative sites in *nrd* repression. *J Bacteriol* **172**, 1711-1718.

Uhlin, U. & Eklund, H. (1994). Structure of ribonucleotide reductase protein R1. *Nature* **370**, 533-539.

Uhlin, U. & Eklund, H. (1996). The ten-stranded beta/alpha barrel in ribonucleotide reductase protein R1. *J Mol Biol* **262**, 358-369.

Uppsten, M., Farnegardh, M., Jordan, A., Eliasson, R., Eklund, H. & Uhlin, U. (2003). Structure of the large subunit of class Ib ribonucleotide reductase from *Salmonella typhimurium* and its complexes with allosteric effectors. *J Mol Biol* **330**, 87-97.

Uppsten, M., Davis, J., Rubin, H. & Uhlin, U. (2004). Crystal structure of the biologically active form of class Ib ribonucleotide reductase small subunit from *Mycobacterium tuberculosis*. *FEBS Lett* **569**, 117-122.

Uppsten, M., Farnegardh, M., Domkin, V. & Uhlin, U. (2006). The first holocomplex structure of ribonucleotide reductase gives new insight into its mechanism of action. *J Mol Biol* **359**, 365-377.

Vassinova, N. & Kozyrev, D. (2000). A method for direct cloning of *fur*-regulated genes: identification of seven new *fur*-regulated loci in *Escherichia coli*. *Microbiology* **146 Pt 12**, 3171-3182.

Voegtli, W. C., Sommerhalter, M., Saleh, L., Baldwin, J., Bollinger, J. M., Jr. & Rosenzweig, A. C. (2003). Variable coordination geometries at the diiron(II) active site of ribonucleotide reductase R2. *J Am Chem Soc* **125**, 15822-15830.

Voevodskaya, N., Narvaez, A. J., Domkin, V., Torrents, E., Thelander, L. & Graslund, A. (2006). Chlamydial ribonucleotide reductase: tyrosyl radical function in catalysis replaced by the FeIII-FeIV cluster. *Proc Natl Acad Sci U S A* **103**, 9850-9854.

Wang, P. J., Chabes, A., Casagrande, R., Tian, X. C., Thelander, L. & Huffaker, T. C. (1997). Rnr4p, a novel ribonucleotide reductase small-subunit protein. *Mol Cell Biol* **17**, 6114-6121.

Williams, S. M., Wing, H. J. & Busby, S. J. (1998). Repression of transcription initiation by *Escherichia coli* FNR protein: repression by FNR can be simple. *FEMS Microbiol Lett* **163**, 203-208.

Wu, R., Zhang, R., Collart, F. & Joachimiak, A. (2003). 1.5A crystal structure of a thioredoxin-like protein NrdI from *Bacillus subtilis*. *To be published*.

Yang, F., Lu, G. & Rubin, H. (1994). Isolation of ribonucleotide reductase from *Mycobacterium tuberculosis* and cloning, expression, and purification of the large subunit. *J Bacteriol* **176**, 6738-6743.

Yang, F., Curran, S. C., Li, L. S., Avarbock, D., Graf, J. D., Chua, M. M., Lu, G., Salem, J. & Rubin, H. (1997). Characterization of two genes encoding the *Mycobacterium tuberculosis* ribonucleotide reductase small subunit. *J Bacteriol* **179**, 6408-6415.

Young, P., Ohman, M. & Sjoberg, B. M. (1994). Bacteriophage T4 gene 55.9 encodes an activity required for anaerobic ribonucleotide reduction. *J Biol Chem* **269**, 27815-27818.

Young, P., Andersson, J., Sahlin, M. & Sjoberg, B. M. (1996). Bacteriophage T4 anaerobic ribonucleotide reductase contains a stable glycyl radical at position 580. *J Biol Chem* **271**, 20770-20775.

Zhao, X., Chabes, A., Domkin, V., Thelander, L. & Rothstein, R. (2001). The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *Embo J* **20**, 3544-3553.

Ziegelhoffer, E. C. & Kiley, P. J. (1995). In vitro analysis of a constitutively active mutant form of the *Escherichia coli* global transcription factor FNR. *J Mol Biol* **245**, 351-361.

9. - Acknowledgments

I recently attended a thesis dissertation and somebody asked the defendant why he had written his thesis in plural, instead of singular, since he was supposed to be defending his only work. I was really shocked by this question and could not help thinking about it for several days. As the reader has probably noticed, this thesis has also been written in plural, since I finally decided that there are so many people that took part in it, in one way or another, that it would not be fair to despise their contribution. Of course, I have not done all the work myself (although most of it), but even if I had, I was not born with the knowledge to design or analyze all the experiments shown here, and definitely not with the ability to "successfully" run all sorts of equipment! The use of the plural, therefore, is my own way to express and endless gratitude to all these people.

It is always a bit harder to write the names of all these guys, since there is a chance that I miss somebody. If I did, please accept my apologies, I swear it was not on purpose!!

First of all I would like to thank Isidre and Eduard, my two supervisors, for their constant and constructive guidance throughout the years. Isidre gave me the opportunity to initiate my Ph.D studies at the Department of Genetics and Microbiology and has always provided me with the freedom to attempt all sorts of experiments, no matter how weird they were. Eduard taught me everything I know about RNRs and molecular biology, plus much more that I never learned!! He has been both an inspiring model and a companion throughout all these years, either in Spain or Sweden.

Our group is a tiny one, and although I learned that the Ph.D is basically a long road that you walk on your own, I could have never made it without those who were always around in the lab and gave me a helping hand whenever I needed one. I am thinking now about Irma, who brought me a whole bunch of plants when I first moved to a new apartment, plants that I never took the time to water until it was too late (I found out the next time she came by!); Núria, whose unpredictable temper introduced an additional excitement to our boring scientists' life, although once you get to know her you find someone with whom you can discuss boring stuff while having a good time. I could not forget Anaïs either; her coming to our lab was a blow of fresh air and lots of laughter, and also a helping hand when I had to

overproduce endless liters of protein and assay β-galactosidase levels, I wish them the best in life!

And then of course there are those who only stayed with us for brief periods of time, but whose contribution is also worth mentioning: Laia, who became an expert constructing *lac*Z fusions; Ester, my second on board for the FNR studies, I will never be able to thank her enough; and Elena, who tried really hard to obtain a *nrd* mutant in *S. pyogenes* and never got the reward that her effort deserved. Thanks a lot to all of them too.

I could not forget my dear friend Oscar either, who has been a loyal companion throughout the years and who developed a special ability to sneak my sequencing samples out of the waiting queue. I cannot help feeling guilty about it!!

I would also like to thank everyone from the Institute of Biotechnology and Biomedicine, those who offered their help and time to overcome the hardships of this job and those who simply contributed to make our lives more pleasant by sharing their leisure time with us (that includes football, paddle, badminton and, of course, "asados").

Almost half of this work has been done in Sweden, at the department of Molecular Biology and Functional Genomics, Stockholm University, where I always had a wonderful time (and I am not talking about the weather!). I am in great debt with Britt-Marie Sjöberg, head of the department and my supervisor up in Stockholm. She guided me through the mysteries of biochemistry and biophysics and took good care of me. I also owe her the suggestion to carry out the heterologous complementation assays (while on a pleasant boat trip over lake Mälaren). I would also like to thank Solveig Hagne, for her patience and dedication during my first stay in Sweden (I know it was not easy for her), Margareta Sahlin, for invaluable help with the EPR and UV-vis experiments and everyone else at the department for always being so nice to me.

Life in Sweden, however, would had never been so exciting if I had not met Alex, Rula or Stef, who became my Swedish family. Besides introducing me to the "fascinating" world of activity assays and protein purification, "Big Boy" Alex turned out to be a great friend. We shared lots of laughter down the corridor or at the coffee room and together with Johanna he contributed to ease my days during the dark Scandinavian winter. Rula gifted me her friendship as well as fascinating coffee-talks about life and people. She and her husband also showed me where to get a good meal around Stockholm and proved excellent cinema

partners!! What can I say about Stef? She was the cutest thing around and had this special ability to cheer me up when I was feeling blue (although she was also able of driving nuts everybody else!). I am very fortunate to have met them all.

I am also very grateful to Adela González de la Campa, June Scott, J.R. Guest, Patricia Kiley and Edmund Lin for kindly providing the strains and plasmids we requested, as well as Cristina and Bryan for a thorough revision of the text.

Finally, this thesis could have never been done without the invaluable support of both Paula and my parents. Paula has suffered with stoicism my ups-and-downs during the last few years and has always managed to keep me going, and my parents have always provided me the best education and their unconditional love. Moltes gràcies!!

RESISTANCE IS FUTILE, YOU WILL BE ASSIMILATED!

THE BORG