CHARACTERISATION OF BACTERIAL NOS

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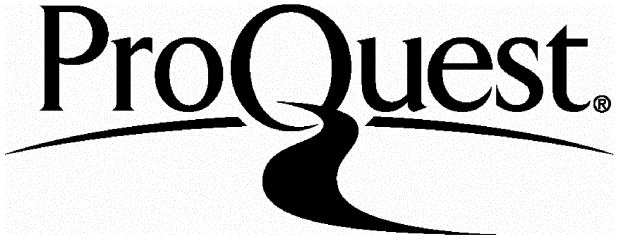

This thesis is dedicated to my wife and our son with love and thanks

ABSTRACT

Nitric oxide (NO) is a pleiotropic regulator of many biological processes, and has been well characterised in eukaryotic systems where it is generated by a family of nitric oxide synthases (NOS). To date, there has been no good molecular evidence for the existence of similar proteins in bacteria. All NOS isoforms characterised so far comprise two distinct domains, an N-terminal heme domain, and a C-terminal reductase domain with sequence similarity to cytochrome P450 reductase. A protein with a high degree of similarity to the N-terminal domain of human inducible nitric oxide synthase (iNOS) has been identified in *Staphylococcus aureus*, and called SANOS. The gene encoding the protein was amplified using PCR, cloned, sequenced, and expressed. The purified protein was characterised using a range of biochemical techniques including heme-spectral analysis, enzyme kinetics, and crystallisation. SANOS is a heme protein, and like mammalian NOS N-terminal domains, possesses the ability to bind L-arginine, the NO intermediate N-hydroxy arginine (NOHA), and NOS inhibitors. Interestingly, SANOS can generate NO from NOHA in a reaction identical to that of the murine iNOS heme domain. The crystal structure of SANOS has been determined as part of a collaboration with Dr. Dave Stammers at Oxford University and shows SANOS to be a dimer. In all the NOS family members studied to date, tetrahydrobiopterin (BH_4) occurs at the dimer interface. Bacteria do not possess BH_4, and interestingly, the crystal structure of SANOS shows the presence of NAD at the dimer interface presumably carrying out the same structural role.

Interestingly, a protein with a high degree of identity to the mammalian NOS C-terminal reductase domain (NADPH cytochrome P450 reductase) was also identified in *S.aureus*. PCR was used to amplify, clone, and express this protein, which was named STAPHRED. Recombinant protein has been generated for enzyme assays in studies with SANOS.

To date, all NOS family members appear as fused heme and reductase domains. This study shows that in *S.aureus* these two domains can be found as separate proteins. This is the first report on the molecular characteristics of NOS-like proteins in bacteria, and illustrates the power of the process from BLAST predictions to the analysis of functional protein.

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ABBREVIATIONS

A	Adenine
ADP	adenosine 5'-diphosphate
Ala or A	Alanine
Arg or R	Arginine
L-Arg	L-arginine
D-Arg	D-arginine
Asn or N	asparagine
Asp or D	Aspartic acid
ATP	adenosine 5'-triphosphate
BH_4	tetrahydrobiopterin
BLAST	Basic Local Alignment Search Tools
Bp	Base pair
BSA	bovine serum albumin
C	Cytosine
°C	degree Celsius
Ca^{++}	Calcium ion
CaCl	Calcium chloride
CAEV	caprine arthritis-encephalitis virus
CaM	calmodulin
CD	cluster determinant
CHO	Chinese hamster ovary
CIAP	calf intestinal alkaline phosphatase
Cm	centimetre
CNS	central nervous system
CO	carbon oxide
COS	An SV_{40} transformed monkey cell line
Cys or C	Cysteine
DAB	diaminobutyric acid
dATP	deoxyadenosine triphophate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate

ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphospates
DTT	Dithiothreitol
dTTP	deoxythymidine triphosphate
EDRF	endothelium derived relaxing factor
EDTA	Ethylene diamine tatra acetic acid
eNOS	endothelial nitric oxide synthase
EPPS	4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid
FAD	flavin adenine dinuleotide
FASTA	a computer tool to compare DNA and protein sequence
FMN	flavin mononucleotide
FPLC	fast protein liquid chromatography
G	Guanine
g	Gram
Gln or Q	Glutamine
Glu or E	glutamic acid
Gly or G	Glycine
GMP	guanosine 5'monophosphate
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HELA	human epidermoid carcinoma cells
His or H	histidine
HPLC	high-performance liquid chromatography
IC_{50}	The median inhibitory concentration
IgG	immunoglobulin G
iNOS	Inducible nitric oxide synthase

IPTG	isopropyl-β -D-thiogalactoside
Ile or I	isoleucine
k	1000
K_2HPO_4	potassium phosphate, dibasic
kb	kilo base(s)
KCl	potassium chloride
kDa	kilo Dalton
KH_2PO_4	potassium phosphate, monobasic
Leu or L	Leucine
L-NAME	N-nitro-L-arginine methyl ester
L-NMMA	N-monomethyl-L-arginine
L-NNA	N-nitro-L-arginine
Lys or K	lysine
M	Molar
Met or M	Methionine
$MgCl_2$	Magnesium chloride
MHC	major histocompatibility complex
mg	Milligram
ml	Millilitre
mM	Millimolar
mRNA	messager ribonucleic acid
M.W.	molecular weight
Na_2HPO_4	Sodium phosphate, dibasic
NaCl	Sodium chloride
NADPH	nicotinamide-adenine dinucleotidephosphate
NaOH	Sodium hydroxide
ng	Nanogram
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide

NOS	nitric oxide synthase
O_2	Oxygen
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PDZ	postsynaptic density-95, Disc Large and Zonula occludentes-1 eukaryotic proteins
pg	Picogram
Phe or F	phenylalanine
PMSF	phenylmethylsulphonyl fluoride
Pro or P	Proline
RNA	ribonucleic acid
rpm	round per minute
SDS	Sodium dodecyl sulphate
Ser or S	Serine
T	Thymine
TBE (10X)	0.9 m Tris.HCL borate pH8.3, 20mM EDTA
TE	10 mM Tris.HCL pH7.5, 1mM EDTA
TEMED	N,N,N',N'-tatramethylethylenediamine
Thr or T	threonine
Tris	tris(hydroxymethyl)aminomethane
Trp or W	tryptoplan
Tyr or Y	tyrosine
μg	microgram
μl	microlitre
μM	micromolar
Val or V	Valine

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CHAPTER 1
INTRODUCTION

CONTENTS

1.1. Overview

Nitric oxide (NO) has been identified as an important molecule in many organisms, including vertebrates and invertebrates. In vertebrates, NO is involved in a number of complex regulatory mechanisms in the brain, the immune network, and the cardiovascular system (Moncada S., *et al.*, 1993).

In the brain and the cardiovascular system, NO participates in neurotransmission and vasodilatation, respectively. The biological effect of NO is transduced via signalling networks involving both transcriptional, and post-transcriptional regulation. One of the most well characterised NO-mediated signal transduction pathways involves the binding of NO to the heme group of soluble guanyl cyclase (sGC), which leads to the production of cyclic GMP (Moncada, S., *et al.*, 1991). In the immune system, macrophages produce NO as part of the oxidative cytotoxic arsenal resulting from macrophage stimulation, typically arising from infection (Poulos, T.L., *et al.*, 1998; Nathan, C. *et al.*, 1994).

Nitric oxide synthases (NOS, EC1.14.13.39) are the enzymes responsible for the generation of NO inside cells. The family of enzymes uses L-arginine (L-arg) as substrate and generates citrulline as a co-product. In mammals, three NOS isoforms have been characterised. The enzymes are all dimeric, bi-domain enzymes that contain iron protoporphyrin IX, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4) as bound prosthetic groups. The three NOS isoforms are: NOS I, neuronal nitric oxide synthase (nNOS, Bredt, D.S., *et al.*, 1991); NOS II, inducible nitric oxide synthase, (iNOS, Lowenstein, C.J., *et al.*, 1992); and NOS III, endothelial nitric oxide synthase, (eNOS, Sessa, W.C., *et al.*, 1992). NOS I and III are calmodulin (CaM)-dependent, and are constitutively expressed in cells, while NOS II is only expressed following immuno-modulation (e.g. in the presence of cytokines and/or bacterial components).

To date the three mammalian NOS isoforms have been intensively studied, and there are many reports on cloning and sequencing, expression, purification, and localisation

of the various isoforms. While there is a large amount of data on eukaryotic NOS isoforms, to date few studies have reported good evidence for a bacterial NOS isoform.

1.2. Research review

1.2.1. NO as a vital molecule of life

NO is a pleiotropic signalling molecule, and has been reported to be involved in a large number of biological roles (Moncada, S., *et al.*, 1993; Nathan, C., *et al.*, 1994). It has a well-established role in the endothelial-dependent control of vascular tone and mediates vascular smooth muscle relaxation by increasing formation of cyclic guanyl monophosphate. NO can also mediate endothelium-independent vascular smooth muscle relaxation in cerebral and other arteries, as well as in the penile corpus cavernosum (Moncada, S., *et al.*, 1991).

In the brain, NO acts as a neural messenger mediating the action of glutamate acting on N-methyl-D-aspartate receptors (NMDA). Recent evidence suggests that NO accounts for a major portion of the neural damage following strokes (Ogura, T., *et al.*, 1993). NO has been suggested to modulate both acute and chronic inflammatory reactions. For example NOS inhibitors have been shown to attenuate acute inflammation in rats with adjuvant arthritis, in lung and skin after immunue-complex mediated injury, and in the bowel after experimental ileitis or colitis. However, other investigators have hypothesised that NO suppresses inflammation by reducing lymphocyte activation (Zhang, J., *et al.*, 1995).

NO administered as a gas relaxes tracheal muscle and reduces methacholine-induced bronchoconstriction. There is increasing evidence that NO functions as the neuro – transmitter of the inhibitory non-adrenergic, non-cholinergic (iNANC) brochodilator response. This may have functional significance because iNANC nerves are the only known neural bronchodilator mechanism in human airways (Barnes, S., *et al.*, 1995). In contrast to its bronchodilator actions, NO is a potent vasodilator and may narrow

airways by dilating bronchial vessels (Robbins, R.A., *et al.*, 1997). Airway blood vessel dilatation and oedema have been proposed to account for the airway obstruction in asthma.

NO may participate in host-defence by mediating anti-microbial activity. NO is critically involved in non-specific and immunological host defence. It has antimicrobial actions against variouse pathogens via its cytotoxic or cytotatic effects. Potent host defence against intruding microbes is also mediated by oxygen radicals and active oxygen species, including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorite anion (OCl^-), produced from phagocyte cells such as neutrophils and activated macrophages. It is now well accepted that the chamical and biological reactivities of NO produced in environments such as inflamed tissue are greatly affected by concomitantly formed oxygen radicals, particularly O_2^-, through formation of reactive nitrogen oxide such as peroxynitrte ($ONOO^-$). These reactive nitrogen and oxygen intermediate species have been documented for host defence reactions against bacteria and fungi (Akaike, T., *et al.*, 2000).

As early as 1989, Stuehr and co-workers had reported that NO was a macrophage product that was responsible for cytostasis and respiratory inhibition in tumour target cells (Stuehr, D.J., *et al.*, 1989). Following that report, other researchers have demonstrated that NO is capable of rapidly and reversible inhibiting the mitochondrial respiratory chain, an effect that may also explain the cytotoxic effects of NO in the CNS and other tissues (Cleeter, M.W., *et al.*, 1994). NO reversibly inhibits oxygen consumption of brain synaptosomal preparations, and this inhibition occurs at the level of cytochrome c oxidase (Brown, G.C., *et al.*, 1994). The research of Clementi, E., *et al.*, (1998) demonstrated that although NO may regulate cell respiration physiologically by its action on complex IV, long-term exposure to NO leads to persistent inhibition of complex I and potentially to cell pathology. NO generated by vascular endothelial cells (under basal and stimulated conditions) modulates the respiration of these cells in response to acute changes in oxygen concentration. Consequently NO plays a physiological role in adjusting the capacity of this enzyme to use oxygen, allowing endothelial cells to adapt to acute changes in their

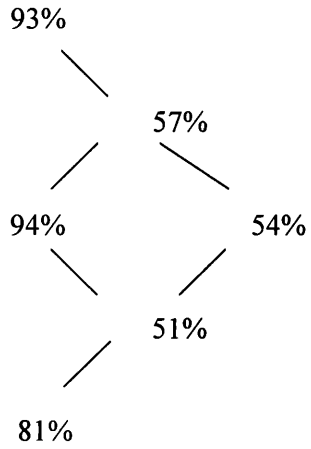
environment. The blockade of complex IV by NO initiates a protective action in the mitochondria to maintain mitochondrial membrane potential (Clementi, E., *et al.*, 1999; Beltran, B., *et al.*, 2000).

1.2.2. The NOS family

In 1991, Bredt *et al.*, reported the first paper on the cloning, sequencing and expression of the cDNA for rat brain NOS (nNOS). The authors concluded that the NOS from rat brain resembled structurally cytochrome P450 reductase. The cDNA encoded an open reading frame of 4230 base pairs, capable of encoding a protein of 1429 amino acids (Bredt, D.S., *et al.*, 1991). During the same year, the groups of Lowenstein and Sessa (1992) published the cloning and expression of macrophage NOS (iNOS) and bovine aortic endothelial NOS (eNOS). The iNOS cDNA was found to possess 3432 base pairs, encoding a protein of 1144 amino acids, while eNOS was found to comprise an open reading frame of 3615 base pairs encoding a protein of 1205 amino acids (Lowenstein C.J., *et al.*, 1992; Sessa W.C., *et al.*, 1992). **Table 1.1** shows the comparison of amino acid identity of the three NOS isoforms (Knowles, R.G., *et al.*, 1994).

The NOS isoforms can be further biochemically classified with respect to calcium/CaM regulation. eNOS and nNOS are classified as calcium/CaM-dependent enzymes while the cytokine inducible form is calcium/CaM-independent. NOS isoforms have been identified in most mammalian species including; human, cow, rat and mouse. Among these four species 39% of the 1144 residues in the shortest isoform are universally conserved. Across the species, the amino acid homology between equivalent isoforms averages 90% (±6%), while within species the homology between different isoforms averages 53% (±2%; Nathan, C., *et al.*, 1994).

Table. 1.1 Comparison of amino acid identity of the three NOS isoforms

Isoforms	Species	mRNA size (kb)	Size of protein (cDNA) (kDa)	Amino acid identity	
				between species	between isoforms
Neuronal (nNOS) Constitutive	Murine	10.5	160.5		
	Human	10.0	161.5		
Endothelial (eNOS) Constitutive	Murine	4.4, 4.8	133.0		
	Human	4.7	133.0		
Macrophage (iNOS) Inducible	Murine	4, 4.5, 5	130.6		
Chondrocyte (iNOS) Inducible	Human	4.4	131.2		

Analysis of the predicted amino acid sequence with the FASTA programme (Pearson, W.R., *et al.*, 1988) revealed very little identity of the N-terminal half (heme domain) of the enzyme to any other known proteins. Some homology of the C-terminal half of NOS (reductase domain) was found with rat cytochrome P-450 reductase (36% identity and 58% similarity over 641 amino acids). The normal function of cytochrome P-450 reductase is to provide cytochrome P-450s with reducing equivalents required for their activity. With the finding of a P-450-type heme moiety in NOS, the same reaction was proposed for this domain (Marletta, M.A., *et al.*, 1993).

In addition, the N-terminal domains of both inducible and constitutive NOS isoforms have been shown to have a maximum absorbance at ~450nm upon reduction and treatment with carbon monoxide. This spectral characteristic is relatively rare and limited to the cytochrome P-450 enzyme family (Marletta, M.A., *et al.*, 1994). The cytochrome P-450 supergene family codes for enzymes, which carry out the oxidative metabolism of endogenous and xenobiotic compounds (Nelson, D.R., *et al.*, 1993). All except one member of the family require a separate flavoprotein reductase and sometimes an iron-sulphur protein to transfer electrons into the heme prosthetic group responsible for the oxidative catalysis. The exception is the fatty acid monooxygenase $P\text{-}450_{BM\text{-}3}$ isolated from *Bacillus megaterium*, whereby a single polypeptide encompasses both the flavoprotein reductase and the heme moiety (Narhi, L.O., *et al.* 1986).

However, although superficially NOS has the appearance of a self-sufficient mammalian P-450 there is no significant homology of the NOS N-terminal sequence with the cytochrome P-450s (Marletta, M.A., *et al.*, 1994).

To date, a large number of eukaryotic NOS isoform sequences have been deposited in the GeneBank, these are summarised in **Table 1.2**.

Table 1.2 NOS sequences in GeneBank.

Species	NOS	Sequence	Author
Bos	eNOS	Complete	Sessa, W.C., *et al.*, 1992
Chicken	iNOS	Complete	Lin, A.W., *et al.*, 1996
Dog	iNOS	Complete	Wang, X., *et al.*, 1998
Dog	eNOS	Complete	Schwemmer, M., *et al.*, 1999
Fly	NOS	Complete	Regulski, M., *et al.*, 1995
Frog	nNOS	Complete	Scheinker, V., *et al.*, 1998
Goat	iNOS	Partial	Adler, H., *et al.*, 1996
Goldfish	iNOS	Partial	Laing, K.J., *et al.*, 1996
Guinea pig	eNOS	Complete	Derst, C., *et al.*, 1999
Guinea pig	iNOS	Complete	Shirato, M., *et al.*, 1998
Horworm	nNOS	Complete	Nighorn, A., *et al.*, 1998
Human	eNOS	Complete	Miyahara, K., *et al.*, 1999
Human	iNOS	Complete	Adams, V., *et al.*, 1998
Human	nNOS	Complete	Fujisawa, H., *et al.*, 1994
Mouse	nNOS	Complete	Ogura, T., *et al.*, 1993
Murine	iNOS	Complete	Lyons, C.R., *et al.*, 1992
Pig	iNOS	Partial	Murtaugh, M.P., *et al.*, 1997
Pig	nNOS	Partial	Smith, A.P.L., *et al.*, 1997
Pond snail	NOS	Complete	Korneev, S.A., *et al.*, 1998
Prolixus	NOS	Complete	Yuda, M., *et al.*, 1996
Rainbow trout	iNOS	Complete	Grabowski, P.S., *et al.*, 1996
Rat	iNOS	Complete	Nunokawa, Y., *et al.*, 1993
Salmon	iNOS	Partial	Oyan, A.M., *et al.*, 1998
Sheep	eNOS	Partial	Aguan, K., *et al.*, 1996
Sheep	nNOS	Partial	Aguan, K., *et al.*, 1996

1.2.2.1. NOS I (nNOS)

The first NOS to be characterised at the molecular level was nNOS, and was isolated from rat brain cerebellar cells (Bredt, D.S., *et al.*, 1991). The cloned nNOS cDNA was 4,287 bases, encoding a protein of 1429 amino acids with a relative molecular mass 160 kDa. Cloning nNOS revealed recognition sites for NADPH, FAD, FMN and CaM as well as phosphorylation sites, indicating that nNOS was capable of being regulated by many different factors. Sequence analysis showed that the only known mammalian enzyme with close homology was cytochrome P450 reductase. Following these studies, nNOS was recognised as a heme binding protein (Klatt, P., *et al.*, 1992), a Ca^{+}/CaM-dependent enzyme (Vorheer, T., *et al.*, 1993), and with a requirement for pterin-binding for full catalytic activity (Klatt, P., *et al.*, 1994). A systematic analysis of nNOS domains was carried out in the Charles laboratory (Lowe, P.N., *et al.*, 1996, Boyhan, A., *et al.*, 1997). The limited proteolysis technique was applied to recombinant rat brain nNOS purified from baculovirus/insect cells. Recombinant nNOS was digested into two parts by trypsin proteolysis, and this cleavage resulted in the loss of CaM binding. From the N-terminus to the C-terminus, the protein comprises binding sites for heme, arginine and BH_4, CaM, FMN, FAD and NADPH. Using the same process, Boyhan, A., *et al.* (1997) located the sites for L-arg and BH_4 binding in the N-terminal domain of rat nNOS. Regions corresponding to amino acids 275-350 and 470-600 were identified as arginine and BH_4 binding sites respectively.

Alignments of the NOS family of proteins demonstrate that the N-terminal sequence of nNOS is longer than those of the other isoenzymes (around 300 residues). Hendriks *et al.,* (1995) described the observation that the nNOS sequence between residues 18 to 97 contains a motif present in several other proteins and variously called 'discs-large homology', 'GLGF repeat,' or 'PDZ domain'. PDZ domains are approximately 100 amino acids in length and the domain is unique to the neuronal isoform of NOS (Hendriks, W., *et al.*, 1995). Some research has suggested that this domain plays a role in determining the interaction of nNOS with other proteins. The PDZ domains are protein-recognition modules. Other proteins that contain PDZ

domain include certain protein kinases and protein tyrosine phosphatases. Many of these PDZ domain-containing proteins are found at specialised cell-cell junctions, such as neuronal synapses and septate junctions. These findings have led to the suggestion that PDZ domains are involved in protein-protein interaction(s) at the plasma membrane (Cho, H.J., *et al.*, 1992). The PDZ containing domain of nNOS has been shown to mediate binding of the enzyme to skeletal muscle syntrophin, a dystrophin-associated protein (Brenman, J.E., *et al.*, 1995).

nNOS contains a PDZ (PSD-95,disc-large and zona occludens-1) domain, a consensus sequence of approximately ninety amino acids that has been shown to mediate protein-protein interactions. In neurons, nNOS is targeted to synaptic sites via its interaction with the PDZ domains of PSD-95 and PSD-93. PSD-95 also interacts with NMDA receptors via one of its PDZ domains. Through its concurrent interaction with nNOS, PSD-95 serves as a physical tether to allow nNOS signalling by NMDA receptor activity. By abolishing expression of PSD-95 protein either by knock out technology in mice or by antisense technology in tissue culture, it has been shown that the presence of PSD-95 is essential for NO production by glutamate stimulation. So the PDZ domain of nNOs serves an important role in helping to localise nNOS to appropriate sites in the neuron (Riefler G.M., *et al.*, 2001).

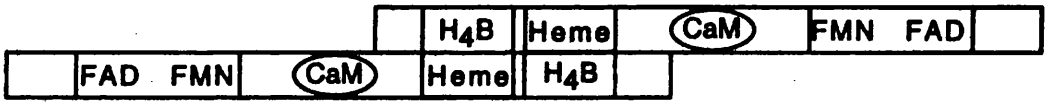
In 1997, NADPH-cytochrome P450 reductase was crystallised successfully and the structure determined (Wang, M., *et al.*, 1997). The C-terminal domain of NOS has been identified as a NADPH cytochrome P450 reductase-like protein (Bredt, D.S., *et al.*, 1991; White, K.A., *et al.*, 1992; Bredt, D.S., *et al.*, 1994; Stuehr, D.J., *et al.*, 1997). The prosthetic group (FMN, FAD and NADPH) binding sites for NOS has been supported not only by the alignment of deduced amino acid sequences, but also by proteolysis and functional analysis (Lowe, P.N., *et al.*, 1996; Gachhui, R., *et al.*, 1996). Therefore, the crystal structure research on cytochrome P450 reductase is important in understanding how these prosthetic groups can function in NOS. **Figure 6.3** shows the alignment of binding motifs in NADPH cytochrome P450 reductase and the rat nNOS domain. The sequences of three prosthetic-group binding sites are conserved compared to other regions between the two proteins. Following the

publication of the sequence of rat nNOS, the sequences of other nNOS cDNAs were published including those for man (Fujisawa, H., *et al.*, 1994), mouse (Ogura, T., *et al.*, 1993), frog (Scheinker, V., *et al.*, 1998), pig (Smith, A.P.L., *et al.*, 1997) and pond snail (Korneev, S.A., *et al.*, 1998).

1.2.2.2. NOS II (iNOS)

The second NOS cDNA to be characterised at the molecular level was inducible NOS (iNOS) from murine macrophage cells (Lowenstein, C.J., *et al.*, 1992). The mRNA used in the cDNA cloning experiments was extracted from LPS and interferon-γ stimulated macrophages. Lowenstein's work, showed that iNOS possesses the recognition sites for FMN, FAD and NADPH and also a consensus CaM binding site. The full-length cDNA is 3432 base pairs, capable of encoding a polypeptide of 1144 amino acids with a calculated molecular mass of 130 kDa. When protein sequence alignments were carried out with rat brain nNOS, a high degree of similarity was noted (Appendix 2). In addition, nNOS is extended at the amino terminus by an additional 200 amino acids and is 15 amino acids residues longer at the carboxyl terminus. Interestingly, iNOS manifests a deletion of 40 amino acids internally.

Using iNOS purified from the RAW 264.7 macrophage cell line, Ghosh, D.K., *et al.*, (1995) proposed a head-to-head subunit interaction model for the iNOS dimer (as shown below).



Trypsin cleaved the subunits of dimeric macrophage iNOS at a single locus, splitting the enzyme into two fragments whose denatured molecular masses were 56 and 73 kDa. The smaller fragments remained dimeric in the native form (112 kDa), contained heme and BH_4, and could bind L-arginine, CO, or imidazole. In contrast, the large fragments were monomeric in their native form, contained FAD, FMN and CaM, and bound NADPH. Although neither purified fragment alone or in combination catalysed the generation of NO from L-arginine, the flavin-containing

fragment did catalyse cytochrome c reduction at a rate that was equivalent to that of native dimeric NOS. These results indicate that trypsin cuts iNOS into two domains that can exist and function independently of one another. The domain that binds heme, BH_4, and substrate is also responsible for maintaining the NOS dimeric structure, while the domain containing FAD, FMN, and CaM is not required for subunit interaction (Ghosh, D.K., *et al.*, 1995).

When site-directed mutagenesis was used to investigate the heme-binding site of human iNOS, cysteine-200 was identified as essential for the dimerisation of heme domains and for the binding of heme, nitroarginine and BH_4 (Cubberley, R.R., *et al.*, 1997). In the same year, the iNOS heme domain was expressed as part of a strategy to characterise the protein residues involved in prosthetic group and dimeric interactions (Ghosh, D.K., *et al.*, 1997). Limited trypsin digestion of the BH_4-free iNOS heme domain cut the protein at a single site in its N-terminal region (K117). BH_4 protected against the cleavage whereas L-arg did not. The resulting 40-kDa protein contained thio-ligated low-spin heme, was monomeric, catalytically inactive, showed no capacity to bind BH_4 or L-arginine, and did not dimerise when provided with these molecules. These results were interpreted as indicating that residues 1-117 were important for iNOS heme domain dimerisation and BH_4/L-arg interaction. A deletion mutant missing residues 1-114 was partially dimeric but otherwise identical to the 40kDa protein regarding its spectral and catalytic properties and inability to respond to L-arginine and BH_4, whereas a deletion mutant missing residues 1-65 was equivalent to wild type iNOS heme domain, narrowing the region of importance to amino acids 66-114. These results suggested that residues 66-114 of iNOS heme domain are involved in BH_4 interaction and subunit dimerisation.

1.2.2.3. NOS III (eNOS)

The third NOS cDNA to be characterised was endothelial NOS, (eNOS). A full-length eNOS clone was isolated by screening a bovine aortic endothelial cell cDNA library using a fragment of rat brain NOS cDNA (Sessa, W.C., *et al.*, 1992). This cDNA has an open reading frame of 3615 nucleotides encoding a protein of 1205-

amino acids, with a calculated molecular mass of 132 kDa. Comparison of the deduced amino acid sequence of eNOS with the nNOS and iNOS sequences reveals 57% and 50% identity, respectively. Functionally eNOS is NADPH and Ca^{+}/CaM-dependent and able to bind L-arginine. In addition eNOS contains a unique N-terminal myristoylation consensus sequence not shared by nNOS or iNOS that may explain its membrane localisation.

Prosthetic site determination studies on eNOS have been carried out by a variety of different research groups. Cysteine 184 of eNOS was recognised as involved in heme coordination and catalytic activity and cysteine 99 shown to be involved in BH_4 binding (Chen, P.F., *et al.*, 1994, 1995). The presence of other eNOS cofactors (FAD, FMN and NADPH) were verified by Venema, R.C., *et al.*, (1997). Experiments with heme-deficient enzyme indicated that the activation of molecular oxygen, and the consequent catalytic reactions, takes place at the heme-containing active site. The oxygenase region also contains the binding sites for L-arginine and BH_4, as well as the necessary determinants for formation of the native enzyme homodimer. This region is therefore the site of the unique catalytic chemistry that results in NO formation, and the lack of strong homology to other proteins suggests that this chemistry requires an equally unique protein structure.

In contrast to the N-terminal half of the NOS polypeptide, the C-terminal half is easily recognisable as an assembly of FAD, FMN, and NADPH binding domains from its sequence similarity to other enzymes (reviewed in Titheradge, M.A., *et al.*, 1998).

In summary, **Figure 1.1** shows a diagram of the domain structure of three NOS isoforms, and **Table 1.3** summarises the NOS isoforms distinctive properties, tissue distribution, subcellular localisation, and the terminology used in the literature to describe the isoenzymes (Titheradge, M.A., *et al.*, 1998). The three isoforms of NOS are all composed of two domains, (N-terminal and C-terminal). There is a sequence in the middle of the two domains corresponding to the binding site for CaM. Located before the CaM site is the N-terminal heme domain (also known as the oxygenase domain) with BH_4, and heme binding sites. After the CaM site in the C-terminal

Figure 1.1 Domain structures of NOS isoforms

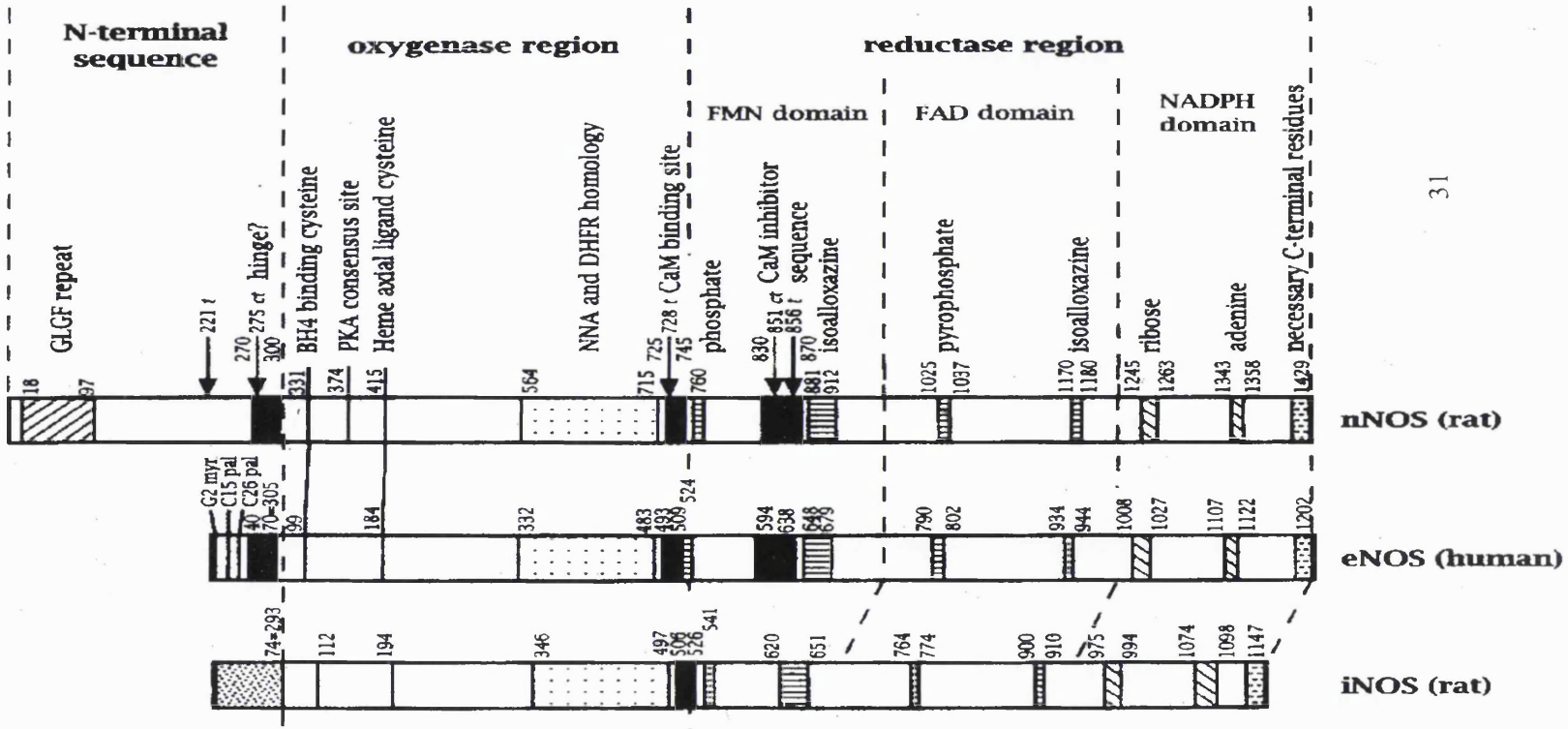


Table 1.3 Human NOS isozymes

NOS Isoenzymes	Alternative description	Human Chromosome	Molecular Mass, kDa	Distinctive properties	Subcellular localisation	Tissue Expression
Neuronal	TypeI nNOS ncNOS bNOS	12	160	Ca^{2+}-dependent constitutively Expressed	Binds to specific proteins via an N-terminal PDZ domain	Neuronal cells Skeletal muscle
Endothelial	Type III eNOS ecNOS	7	134	Ca^{2+}-dependent constitutively expressed	Targets to the Golgi and to calveoli via N-terminal Myristoylation and palmitoylation	Endothelial cells Epithelial cells cardiomyocytes
Inducible	Type II iNOS macNOS	17	130	Ca^{2+}-independent induced by inflammatory stimuli (cytokines,LPS)	Soluble?	Macrophages Hepatocytes Astrocytes Smooth muscle Cells (and many more)

domain is the region with considerable sequence identity to cytochrome P450 reductase. This domain has an orderly arranged series of binding sites for FMN, FAD and NADPH.

Additionally, in nNOS, there is an extra N-terminal sequence of around 200 amino acids not present in the other isoforms. Both N-and C-terminal domains are necessary for fully functional NOS activity.

1.2.3. Expression of NOS

The recombinant expression of functional NOS has played an important part in NOS research throughout the 1990's. NOS isoforms have been expressed in mammalian cells, insect cells, yeast and *E. coli.*

The first recombinant NOS to be studied were rat brain nNOS (Bredt, D.S., *et al.*, 1991). The cDNA encoding nNOS was expressed in human kidney 293 cells (HK293). The recombinant expression was verified by antibody staining, paralleled with three basic NOS activity assays: (i) citrulline formation, (ii) L-arginine oxidation to nitrite, and (iii) the enhancement of endogenous guanylyl cyclase activity in response to newly synthesised NO (Bredt, D.S., *et al.*, 1991). Using this cell line, the same group expressed murine macrophage iNOS and identified NOS enzyme activity within the cell lysate. The third NOS isoform, eNOS, was expressed in the mammalian cell line COS1 in 1992 (Sessa, W.C., *et al.*, 1992). For this enzyme, the membrane fraction of the transfected cells presented the citrulline formation activity, suggesting that eNOS was membrane associated.

All three NOS isoenzymes had been expressed by 1992, but at that time the expression was not at a high level and the protein could not be purified for multifunctional assays, or crystallisation studies.

Following the expression of rat brain nNOS in mammalian cells, it was expressed in the baculovirus/insect cell system and purified with 2'-5'ADP-sepharose-4B. The

recombinant nNOS was expressed at very high levels, was soluble, and had NOS enzymatic activity, which could be inhibited by a panel of NOS inhibitors (Charles, I.G., *et al.*, 1993). **Table 1.4** shows the expression data of NOS isoforms in different systems.

The first report of NOS isoform expression in *E. coli* was in 1995, when McMillan, K., *et al.* expressed separate domains of rat brain nNOS. The authors reported the successful use of the vectors pCWori, and pPROK-1. During the expression of the oxygenase domain, the heme precursor δ-aminolevulinic acid was added to the culture medium to 0.5 mM final concentration. The two domains were purified and their activity tested. The partialy purified heme protein elicted a reduced CO-difference spectrum with a wavelength maximum at 445 nm. The samples exhibited spectral perturbations upon addition of L-arginine, characterised by a 395 nm wavelength maximum and a 420 nm minimum in the difference spectrum (type I; McMillan, K., *et al.*, 1993). These properties indicate that the bacterially expressed hemoprotein possesses properties of the intact NOS enzyme.

The bacterial expression of the NOS heme-binding oxygenase and flavinprotein oxidoreductase domains as isolated proteins with specific properties of the intact enzyme represented an important development in structure-function studies of this complex enzyme (McMillan, K., *et al.*, 1995).

In 1996, two groups of researchers expressed human eNOS and mouse iNOS in *E. coli* with co-expression of CaM (Rodriguez-Crespo, I., *et al.*, 1996; Wu, C., *et al.*, 1996). Using this co-expression system, the resulting NOS enzymes were not only expressed at a higher level, but also showed higher enzyme activity. It was found that co-expression of human CaM with human eNOS improved the yield of the hemoprotein approximately threefold. Co-expression also produces a higher quality protein, as indicated by an increase in its catalytic activity from 100-130 nmol.min-1.mg-1 for the non-co-expressed protein to 140-170 nmol.min-1.mg-1 for the co-expressed protein. Likewise, the cytochrome c reduction activity increased modestly from 1.5 (±0.3) to 1.8 (±0.3) μmol.min-1.mg-1 when the protein is co-expressed with

Table 1.4 Expression of recombinant NOS isozymes

Isoenzyme	Species	Expression	References
nNOS	Human	Baculovirus	Charles, I.G., *et al.*, 1993
	Human	COS cells	Nakane, M., *et al.*, 1993
	Human	Baculovirus	Nakane, M., *et al.*, 1995
	Rat	Baculovirus	Richards, M.K., *et al.*, 1994
	Rat	293 cells	Bredt, D.S., *et al.*, 1991
	Rat	*E. coli*	McMillan, K., *et al.*, 1995
	Rat	Yeast	Black, S.M., *et al.*, 1995
eNOS	Bovine	COS cells	Sessa, W.C., *et al.*, 1992
	Bovine	Baculovirus	Busconi, L., *et al.*, 1995
	Bovine	*E. coli*	Mastasek, P., *et al.*, 1996
	Human	3T3 cells	Janssens, S.P., *et al.*, 1992
	Human	Baculovirus	Chen, P.F., *et al.*, 1996
	Human	*E. coli*	Rodriguez-Crespo, I., *et al.*, 1996
iNOS	Human	HELA	Charles, I.G., *et al.*, 1993
	Human	CHO cells	Laubach, V.E., *et al.*, 1996
	Human	293 cells	Geller, D.A., *et al.*, 1994
	Human	Baculovirus	Nakane, M., *et al.*, 1995
	Mouse	*E. coli*	Fossetta, J.D., *et al.*, 1996
	Mouse	Baculovirus	Xie, Q.W., *et al.*, 199
	Mouse	Yeast	Sari, M.A., *et al.*, 1992
	Rat	293 kidney cells	Karlsen, A.E., *et al.*, 1995
	Rat	COS cells	Adachi, H., *et al.*, 1993

Note: Data from Titheradge M A (1998).

CaM. These gains in yield and activity are not obtained if excess CaM is simply added to non-co-expressed eNOS. It is clear that CaM protects eNOS, or otherwise facilitates production of the properly folded, active protein (Rodriguez-Crespo, I., *et al.*, 1996). These results suggest that the functional expression of NOS needs the presence of the important co-factor CaM while protein translation occurs.

For recombinant eNOS expressed in *E. coli*, (Martasek, P., *et al.* 1996), a 90% purification was achieved using 2,5'-ADP Sepharose-4B column chromatography. The recombinant protein appeared as a single band of molecular mass 135 kDa on SDS-PAGE, in good agreement with the previously reported molecular mass for native eNOS and for the enzyme obtained from a baculovirus expression system. Western blot analysis demonstrated eNOS immunoreactively against a 135-kDa protein band from the *E. coli* cytosol, which was indistinguishable on SDS-PAGE from bovine eNOS expressed in HEK 293 cells. A small portion of the enzyme expressed in *E. coli* was also present also in the pellet fraction. This insoluble protein was unlikely to be due to membrane association (due to N-myristoylation of eNOS), because *E. coli* does not contain the transferase required for N-myristoylation (Martasek, P., *et al.*, 1996). *E. coli* expressed iNOS appears to have the same specific activity as murine iNOS, a similar Km for arginine binding, and similar IC_{50} values for three iNOS inhibitors as does mammalian-derived iNOS. These experiments support the hypothesis that no mammalian-specific modification(s) of iNOS are necessary for NOS activity (Fossetta, J.D., *et al.*, 1996).

The functional expression of recombinant NOS, and the purification of the resulting protein has been a fundamental task in the study of the biochemistry of NOS. Although the NOS heme domain has been crystallised, the full-length NOS (with full catalytic activity) remains uncrystallised, and is still a big challenge to NOS researchers.

1.2.4. NOS catalytic activity and regulation

The overall reaction catalysed by NOS is shown in **Figure 1.2** (reviewed in Titheradge, M.A., *et al.*, 1998). NOS enzymes are catalytically dependent on reducing equivalents derived from reduced nicotinamide adenine dinucleotide phosphate (NADPH), and require molecular oxygen as a substrate. The conversion of L-arginine to L-citrulline with co-production of NO takes place in two steps with N^G-hydroxyl-L-arginine (NOHA) as an intermediate. Two moles of O_2 and 1.5 moles of NADPH are consumed per mole of formed products. The NOS reaction mechanism has been discussed recently in reviews (Griffith, O.W., *et al.*, 1995; Marletta, M.A., *et al.*, 1994). The two reaction steps are catalysed by heme iron (Pufahl, R.A., *et al.*, 1993). The first step of L-arginine hydroxylation is formally equivalent to a typical cytochrome P450 monoxygenase reaction. The second step involving 1-electron oxidation of the intermediate NOHA is unusual but not unique to catalyse oxidation of NOHA and related guanidines to NO and the corresponding urea derivative.

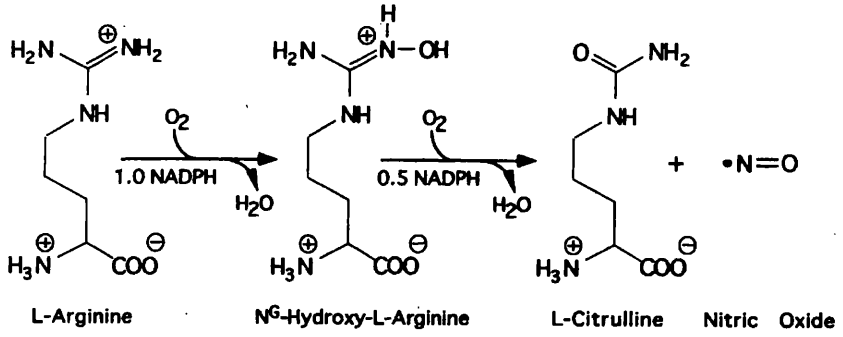


Figure 1.2 The NOS reaction.

The NO synthases use L-arginine as their substrate to produce NO and L-citrulline (Knowles, E.S., *et al.*, 1989; Palmer R.M., 1989; Stuehr D.J., *et al.*, 1989). The NO synthases are flavoproteins containing bound FMN and FAD. They are dependent on NADPH and O_2 as co-substrates, and require BH_4, CaM and heme for enzymatic activity. The conversion of L-arginine to L-citrulline and NO by NOS is known to involve two separate monooxygenase steps (Knowles, E.S., *et al.*, 1990; Stuehr, D.J., *et al.*, 1991). The first step involves the oxygenation of L-arginine to N-hydroxyarginine. This reaction is followed by the oxygenation of N-hydroxy-L-arginine to L-citrulline and NO. The reaction requires two molecule of O_2, one and

half NADPH and the presence of BH_4. The reaction mechanism shows some similarities to reactions carried out by the aromatic amino acid hydroxylases, which also utilise BH_4 and the cytochrome P450.

The affinity of NOS for BH_4 has been shown to increase six-fold in the presence of 0.1 mM L-arginine, and in a similar manner, BH_4 increases NOS affinity for L-arginine. Thus NOS shows interdependence for substrate and BH_4 binding. These results indicate that the NOS BH_4 binding site allosterically interacts with the binding site for L-arginine and presumably also with the heme. Further studies on the interaction of the tightly bound substrate, and the BH_4 binding domain with the heme moiety within the catalytic centre of the enzyme may reveal the precise role of BH_4 in the catalytic reaction (Klatt, P., *et al.*, 1994).

BH_4 has been proposed to maintain NOS in an active configuration. This possible role for BH_4 comes from the finding that NOS isolated in the absence of BH_4 is very unstable, upon reduction and binding of CO the unstable NOS converts from a species exhibiting a Soret absorption maximum of 443nm to a species with a Soret maximum of 421 nm. The heme in this form of the enzyme is co-ordinated by a weaker ligand than the thiolate ligand of cysteine, consistent with histidine co-ordination in the CO bound form of 421nm. These data suggest that BH_4 plays an important role in stabilisation of NOS (Wang, M., *et al.*, 1995).

The two domains of murine iNOS were used to reconstitute NOS activity successfully in 1995 (Ghosh, D.K., *et al.*, 1995). The two domains were isolated following trypsin digestion. Mixing the domains at various ratios showed that NO could not be produced from L-arginine, but could be formed from the reaction intermediate NOHA. The apparent Km with NOHA in the reconstituted system was 100 μM versus 19 μM for native iNOS. Between 2.5 and 3.0 moles of NADPH were consumed per mole of NO formed from NOHA, considerably higher than the stoichiometry obtained with native iNOS (0.5 NADPH oxidised per NO formed), indicating an uncoupled electron transfer between the domain fragments. Thus, the isolated iNOS reductase and oxygenase domains each retain their separate catalytic

function, but interact to catalyse only the second step of NO synthesis (Ghosh, D.K., *et al.*, 1995). In the following year, Chen, P.F., *et al.* (1996) reconstituted human eNOS activity by mixing two domains, which were expressed in the baculovirus/insect cell system. By contrast, this study did not report the reconstituted system needed NOHA as substrate, and demonstrated that arginine itself could be used as a substrate. The activity measured in the reconstituted system was clearly present, but at lower levels, corresponding to only one twentieth of the native eNOS.

This is an important paper as far as the evolutionary study of NOS is concerned, for although the two separate domains of NOS had lower activity, they could still reconstitute enzymatic activity when necessary co-factors and substrates were provided.

Paralleled with the mechanistic studies, NOS biology has been studied by the use of specific NOS inhibitors. The most commonly used inhibitor is N-monomethyl-L-arginine (L-NMMA), which competes with L-arg, and is capable of inhibiting all the NOS isoforms. L-NMMA was the inhibitor used to investigate the cytotoxic affects of macrophages, and it enabled the cytotoxic element to be identified as NO (Hibbs, J.B., *et al.*, 1987; Stenger, S., *et al.*, 1995; Fukatsu, K., *et al.*, 1996). Other inhibitors include imidazole (Wolff, D., *et al.*, 1993a, b; 1994a,b), thiocitrulline (Abu-Soud, H.M., *et al.*, 1994), N-nitroarginine (Furfine, E.S., *et al.*, 1993; Rondouin, G., *et al.*, 1993; Klatt, P., *et al.*, 1994; Alderton, W.K., *et al.*, 1998), N-(3-(aminomethyl) benzyl) acetamidine (1400W) (Garvey, E.P., *et al.*, 1997; Thomsen, L.L., *et al.*, 1997; Hamilton, L.C., *et al.*, 1998), S-ethylisothiourea (S-EITU) (Garvey, E.P., *et al.*, 1994; Nakane, M., *et al.*, 1995; Southan, G.J., *et al.*, 1996), and N^6-iminoethyl-L-lysine (L-NIL) (Southan, G.J., *et al.*, 1996; Wolff, D.J., *et al.*, 1998; Grant, S.K., *et al.*, 1998; Handy, R.L.C., *et al.*, 1998). These studies showed not only the regulation of NOS activity, but also the different properties of NOS isoenzymes.

1.2.5 NOS crystallisation

Recently, several papers have published reports on NOS crystallisation. A fragment containing amino acids 114 to 498 of iNOS was reported in 1997 (Crane, B.R., *et al.*, 1997). This domain of NOS is variously referred to as the heme domain, or the oxygenase domain (NOS_{OX}). The crystal structure of NOS_{OX} revealed an unusual fold and heme environment for stabilisation of activated intermediates key for catalysis.

The authors described the resulting heme binding fold of curved α-β domains as a 'baseball catcher's mitt' with the heme clasped in the palm of the mitt. The location of exposed hydrophobic residues, and the results of mutational analysis placed the dimer interface adjacent to the heme-binding pocket. Juxtaposed hydrophobic O_2- and polar L-arginine binding sites were occupied by imidazole and aminoguanidine, respectively. This structural information is important in providing a template for designing NOS inhibitors that may be useful in a variety of pathological situations.

The following year, the crystallisation of another iNOS heme-domain (amino acids 66 to 498) was reported by the same group (Crane, B.R., *et al.*, 1998). This work described the crystal structure of the murine iNOS oxygenase dimer, with active-centre water molecules. The presence of both the substrate L-arginine, (or product analogue thiocitrulline) and the cofactor BH_4, completed the catalytic centre for synthesis of NO. BH_4-binding appears to refold the central interface region of the dimer, and creates a 30-angstrom-deep active-centre channel important for catalysis (Crane, B.R., *et al.*, 1998).

Reports on the crystallisation of the eNOS heme domain were published in 1998 and 1999 (Raman, C.S., *et al.*, 1998; & Fischmann, T.O., *et al.*, 1999). In their research, the phylogenetically conserved Cys-$(X)_4$-Cys motif and its strategic location established a structural role for the metal centre in mammalian NOS heme binding. The integrity of the BH_4-binding site revealed an unexpected structural zinc atom

situated at the intermolecular interface and co-ordinated by four cysteines, two from each monomer.

1.2.6 NOS localisation

In addition to the characterised NOS isoforms reported in GeneBank, NOS has also been found in many species by using anti-NOS antibodies or NADPH-diaphorase histochemistry.

In vertebrates, NOS positive neurones have been found in different cell layers of the brain, and of the retina. Studies have been reported for tiger (Kurenni, D.E., *et al.*, 1995), turtle (Haverkamp, S., *et al.*, 1998), rat (Haverkamp, S., *et al.*, 1998), guinea pig, mouse, cat and rabbit (Kim, I.B., *et al.*, 1999) and pig (Meyer, P., *et al.*, 1999). The role of NO in the visual system was suggested to be very important from biochemical studies in light transduction and signal transmission (Haverkamp, S., *et al.*, 1998). In the porcine system, NOS positive cells were located in the ciliary epithelial and trabecular endothelial cells. These results indicate that nNOS might be involved in both the production and outflow of aqueous humour in pig eyes (Meyer, P., *et al.*, 1999).

Most of the reports of nNOS localisation have been found from the central nervous system. NOS-positive neurones have been reported in the central nervous system of various animals such as rainbow trout brain (Soderstrom, V., *et al.*, 1995), frog brain (Bruning, G., *et al.*, 1996), goldfish CNS (Bruning, G., *et al.*, 1995), ferret brain (Matsumoto, T., *et al.*, 1992), cat spinal cord (Pullen, A.H., *et al.*, 1997), Swordtail fish spinal cord (Anken, R.H., *et al.*, 1996), dog spinal cord (Vizzard, M.A., *et al.*, 1997), macaque CNS (Satoh, K., *et al.*, 1995), teleost spinal cord (Cioni, C., *et al.*, 1997), and newt olfactory bulb (Bruning, G., *et al.*, 1996). Studies on blood circulation in turtle suggested that there is a NO-dependent vasodilatory tonus affecting both cerebral and systemic blood circulation (Soderstrom V., *et al.*, 1995). The presence of NOS in projection areas of most afferent nerves suggested a widespread involvement of NO in sensory information processing in goldfish

(Bruning G., *et al.*, 1995). NO may function as a transmitter in thoracolumbar sympathetic preganglionic neurones, but not in sacral parasympathetic preganglionic neurones in dog brain (Vizzard MA., *et al.*, 1997). From NOS immunocytochemistry studies in macaca fuscata, (Satoh, K., *et al.*, 1995) it was apparent that there was a species difference in the distribution of central NOS-containing neurones, although the consequences (and physiology) arising from these differences are not clear.

In addition to neuronal tissue, some other tissues were also found to be NOS positive by immuno-staining. For example, gut neurones in Atlantic cod (Karila, P., *et al.*, 1997), lamprey olfactory mucosa (Zielinski, B.S., *et al.*, 1996), hamsters vascular and skeletal muscle cells (Segal, S.S., *et al.*, 1999), goat bone joints (Lechner, F., *et al.*, 1999), bullfrog heart (Clark, R.B., *et al.*, 1994), pancreas neurones of mouse, rat, chicken, kitten and monkey (Liu, H.P., *et al.*, 1994). In the lamprey olfactory mucosa, (Zielinski, B.S., *et al.*, 1996) light and electronic microscopy was used to investigate the distribution of NOS positive cells. In this system, NO modulates perireceptor events following L-arginine chemostimulation, resulting in olfactory receptor cell axonal activity.

A goat model has been used to study the role of iNOS in arthritis development, (Lechner F *et al.* 1999). Most iNOS positive cells expressed neither MHC class II nor CD68, which suggests that they were fibroblast like synoviocytes. *In situ* hybridisation studies showed that there was no correlation between iNOS immune-reactivity and detectable virus expression in the joint. In addition, infection of macrophages *in vitro* did not lead to increased iNOS mRNA expression. In response to stimulation, similar levels of iNOS expression were observed in infected and uninfected macrophages. These findings suggest that the expression of iNOS is a feature of late-stage chronic arthritis and is not involved in the development of the inflammatory lesions.

In invertebrates, NOS staining positive cells have been found in a wide variety of organisms. For example: in squid CNS (Kimura, T., *et al.*, 1997), in the pond snail nervous system (Serfozo, Z., *et al.*, 1998), in locust brain (Ott, S.R., *et al.*, 1998), in

leach CNS (Leake, L.D., *et al.*, 1996), in sea slugs (Moroz, L.L., *et al.*, 1996), in medfly CNS (Conforti, E., *et al.*, 1999), and in cuttlefish CNS (Palumbo, A., *et al.*, 1999).

It is surprising that NOS immuno-positive cells have also been reported in plants for example in maize cells (Ribeiro, E.A., *et al.* 1999).

Table 1.5 summarises the findings of NOS antibody immunolocalisation studies. As shown in **Table 1.5**, NOS isoforms have been reported in a vast range of organisms.

1.2.7 NOS in bacteria

NOS isoforms have been found in many eukaryotic organisms and have been characterised at both the genetic and biochemical levels. Although there have been reports of the biochemical identification of NOS in bacteria, at the time of writing this thesis, there has been no definitive genetic characterisation of a functional bacterial NOS gene.

Two groups have however reported the finding of bacterial NOS activity. The first report was from *Nocardia sp.* (Chen, Y., *et al.*, 1994, 1995). In their study, the cell lysate and supernatant of *Nocardia sp.* were used to measure NOS activity. The fraction with NOS activity (as measured by the radioactive citrulline assay) was concentrated and later purified. The citrulline formation required NADPH, O_2, Ca^{++}, FAD, FMN and BH_4, and could be inhibited by an NOS inhibitor N^G-hydroxy-L-arginine. The purified NOS from *Nocardia sp.* was shown to have an apparent molecular mass of 51.9 kDa as determined by SDS-PAGE. This is much smaller than characterised full-length mammalian NOS isoforms, which have molecular masses of around 130-160 kDa. Although twelve amino acids were sequenced from the N-terminus of the *Nocadia* NOS-like protein, no matched sequences were found in SwissProt, GenPept, GeneBank, and EMBL database using BLAST and TBLASTN programs.

Table 1.5 The localisation of NOS isoforms in different species

Species	NOS	Localisation	Author
Atlantic cod	nNOS	Gut neurones	Karila, P., *et al.*, 1997
Bullfrog	nNOS	Heart	Clark, R.B., *et al.*, 1994
Cat	nNOS	Spinal cord	Pullen, A.H., *et al.*, 1997
Chick	NOS	Pancreas neurons	Liu, H.P., *et al.*, 1994
Dog	nNOS	Spinal cord	Vizzard, M.A., *et al.*, 1997
Ferret	nNOS	Brain	Matsumoto, T., *et al.*, 1992
Frog	nNOS	Brain	Bruning, G., *et al.*, 1996
Goat	iNOS	Joints	Lechner, F., *et al.*, 1999
Goldfish	nNOS	CNS	Bruning, G., *et al.*, 1995
Guinea pig	NOS	Retina	Kim, I.B., *et al.*, 1997
Hamsters	NOS	Vascular/muscle	Segal, S.S., *et al.*, 1999
Kitten	NOS	Pancreas neurones	Liu, H.P., *et al.*, 1994
Lamprey	nNOS	Olfactory mucosa	Zielinski, B.S., *et al.*, 1996
Leech	nNOS	CNS	Leake, L.D., *et al.*, 1996
Locust	nNOS	Neurones	Ott, S.R., *et al.* 1998
Macaque	nNOS	CNS	Satoh, K., *et al.*, 1995
Maize	NOS	Root, leaves	Ribeiro, E.A., *et al.*, 1999
Medfly	NOS	CNS	Conforti, E., *et al.*, 1999
Monkey	NOS	Pancreas neurones	Liu, H.P., *et al.*, 1994
Mouse	NOS	Pancreas neurones	Liu, H.P., *et al.*, 1994
Newt	nNOS	Olfactory bulb	Bruning, G., *et al.*, 1996
Pond snail	nNOS	Nervous system	Serfozo, Z., *et al.*, 1998
Porcine	NOS	Ocular tissue	Meyer, P., *et al.*, 1999
Rabbit	NOS	Retina	Kim, I.B., *et al.*, 1997
Rainbow trout	nNOS	Brain	Soderstrom, V., *et al.*, 1995
Rat	NOS	Pancreas neurones	Liu, H.P., *et al.*, 1994
Rat	nNOS	Retina	Haverkamp, S., *et al.*, 1998
Sea slug	NOS	CNS	Moroz, L.L., *et al.*, 1996
Sepia	nNOS	CNS	Palumbo, A., *et al.*, 1999
Swordtail fish	nNOS	Spinal cord	Anken, R.H., *et al.*, 1996
Squid	NOS	CNS	Kimura, T., *et al.*, 1997
Teleost	nNOS	Neuronsecretory	Cioni, C., *et al.*, 1997
Tiger	nNOS	Retina	Kurenni, D.E., *et al.*, 1995
Turtle	nNOS	Retina	Haverkamp, S., *et al.*, 1998

The other group to publish on bacterial NOS demonstrated NOS activity in cell lysates and the culture medium of *Staphylococcus aureus* (Choi, W., *et al.*, 1997, 1998). They tested the cell lysate and culture medium using the radioactive NOS enzyme assay, with measurement of L-citrulline formation. Interestingly, anti-rat and anti-mouse iNOS IgG antibodies were used to analyse the protein in Western blotting experiments. These studies identified a 55-kDa protein band that cross-reacted with the antibodies. Overall, although this study demonstrated measurable L-citrulline generation, the NOS protein was not isolated from the bacteria, and no further characterisation was carried out afterwards.

The first report of a bacterial NOS-like DNA sequence was in *B. subtilis* (Kunst, F., *et al.*, 1997). The only clue of the NOS-like sequence came from the *B. subtilis* genome annotation, where the gene named *yflM*, was characterised as having similarity to NOS (Appendix 4).

The above reports suggested that bacteria might possess NOS-like proteins, similar to those found in mammals. The objective of this thesis was to characterise bacterial proteins responsible for NO generation.

1.3. The necessity of the study

The enzymology and biochemistry of NOS has been intensively studied during the past twenty years. Studies on the enzymological catalysis, activity regulation, genomic localisation, and the functional inhibition of NO-generation have demonstrated the importance of NO in disease and health. However, compared to studies on eukaryotic NOS isoforms, there have been very few studies on bacterial NOS.

As NO has been demonstrated to play a vital role in many physiological and pathological signal transduction pathways in eukaryotic biology, it may also be important in bacteria. The study of NOS in bacteria may shed light on biological processes important in bacterial signalling pathways.

1.4. Purpose of the study

The purpose of the project was to characterise NOS-like sequences in bacteria. The project was aimed at identifying bacterial NOS-like genes, and cloning them with the aim of generating recombinant bacterial NOS protein for further study.

The work reported in this thesis was carried out at the Wolfson Institute for Biomedical Research, University College London, with some biochemical experiments carried out at GlaxoWellcome, Stevenage. Highly purified protein samples for crystallisation studies were made at Newcastle University, and the crystallisation and X-ray structural data was generated at Oxford University.

CHAPTER 2
MATERIALS AND METHODS

CONTENTS

2.1. Overview

The work in this thesis was carried out in the Molecular Biology Laboratory, Wolfson Institute for Biomedical Research, University College London, and supported by a Studentship from GlaxoWellcome PLC.

2.2. General materials

2.2.1. General chemicals

Ordinary chemicals were obtained from the Sigma chemical company or BDH, unless otherwise stated.

2.2.2. DNA restriction enzymes

Restriction endonucleases were purchased from Roche.

2.2.3. DNA modification enzymes

The *Pfu* DNA polymerase was from Stratagene; Alkaline phosphatase, *Taq* DNA polymerase, and T_4 DNA ligase were obtained from Roche.

2.2.4. Oligonucleotides

Oligonucleotides were synthesised by Genosys, Sigma.

2.2.5. DNA sequencing reagents

T_7 Sequenase V 2.0 reagents were from Amersham Life Science; GEL-Mix6 DNA sequencing gel, (GIBCO BRL) (5.7% Acrylamide, 0.3% N, N'-Methylene-biscrylamide, 7M Urea, 100mM Tris-borate pH8.3, 1mM Na_2EDTA, 3 mM TEMED), dNTP (Roche).

2.2.6. Radioactivity

All radioactive reagents were purchased from Amersham, for DNA sequencing: [α-^{35}S] dATP 10 mCi/ml, >1000 Ci/mmol; for Northern blotting: [α-^{32}P] dCTP 10 mCi/ml, 3000Ci/mmol.

2.2.7. RNA Northern blot reagents

Hybond-N Nylon membrane optimised for nucleic acid transfer was from Amersham. RNeasy Mini kits were from QIAGEN; NorthernMax™ Northern Blotting Kit was from Ambion.

2.2.8. Protein analysis, purification reagents and apparatus

For protein analysis, SDS-PAGE and protein blots were performed using the Mini PROTEAN II system (BIO RAD). For his-tagged protein purification, TALON™ Superflow™ affinity Resin was from Clontech. For GST fusion protein purification, Glutathione Sepharose™ 4B was used, and was supplied by Amersham.

The TALON column was set-up in the FPLC UPC-900 system (Amersham Pharmacia biotech).

2.2.9. Western blot reagents

DAKO liquid DAB was supplied by the DAKO Corporation. ECL Western blotting detection reagents, Hybond-ECL membrane and Hyperfilm ECL were obtained from Amersham.

2.2.10. Spectrophotometry equipment

The UV-2401 PC, UV-VIS Recording spectrophotometer was supplied by Shimadzu. The Lambda 7 UV/VIS spectrophotometer was supplied by Perkin Elmer.

2.2.11. H_2O_2 shunt assay reagents

Superoxide dismutase and BSA (10 mg/ml) was from Sigma. Greiss reagent A (1 g sulphanilamide in 2.94 ml concentrated phosphoric acid, made up to 100 ml with distilled water). Greiss reagent B (0.5 g NEDA in 2.94 ml concentrated phosphoric acid, made up to 100 ml with distilled water).

2.2.12. Heme determination reagents

5 M NaOH; Pyridine; Sodium Dithionite.

2.3. Stock solutions

30% Acrylamide/Bis solution (BIO RAD)
100 mM δ-aminolevulinic acid
10% Ammonium persulfate
1M DTT
0.5M EDTA
1M EPPS, pH7.5
10mg/ml Ethidium bromide
1M Hepes, pH 7.5
100 mM IPTG
PBS: 0.8 g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1 litre sterile water
10 mM PMSF
3 M Sodium acetate pH4.8
5 M NaCl
10% SDS
TE buffer (10 mM Tris, 1 mM EDTA, pH8.0)
Tris solutions in 1.5 M, pH8.8; 1 M pH6.8; 1M pH7.5
8 M urea
100 mM NP-40
10%(w/v) CHAPS

10% (w/v) DDMAU

10%(v/v) Triton-X100

0.5mM n-DODECYL β -D-Maltoside

1 M n-Octyl-β-D-glucopyranoside.

2.4. Bacteriological media and antibiotics

All microbiological growth mediums were autoclaved at 120b/in^2 for 20 minutes after they were made.

2.4.1. Terrific broth (TB)

1.2%(w/v) tryptone, 2.4%(w/v) yeast extract, 0.4%(v/v) glycerol made up to 900 ml with sterile water and autoclaved. After cooling, 100 ml of sterile 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, pH 7.5 was added to make up to 1,000 ml. This medium was the growth medium of choice for the expression of recombinant protein in *E. coli.*

2.4.2. Luria-Bertani broth (LB)

1% (w/v) tryptone, 0.5%(w/v) yeast extract, 171 mM NaCl, for agar plates, this was supplemented by the addition of 1.5% bacto agar, and adjusted to pH 7.5 by the addition of 10M NaOH. This medium was used for growth of *E. coli* where plasmid purification was to be carried out, and for *B. subtilis* culture.

2.4.3. 2×YT broth

1.6% (w/v) tryptone, 1% (w/v) yeast extract, 171 mM NaCl, adjust pH to 7.4 by the addition of 5 M NaOH. This medium was used for expression of recombinant proteins in *E. coli.*

2.4.4. M9 minimal medium

10×M9 salts: Na_2HPO_4 60g, KH_2PO_4 30g, NaCl 5g, NH_4Cl 10g, add water to 1000 ml then sterilise by autoclaving. 20% (w/v) glucose separately prepared and was filtered sterilised. To make M9 minimal medium: 10×M9 salts (98.8 ml), 20% (w/v) glucose (10 ml), 1M $MgCl_2$ (2 ml), 1M $CaCl_2$ (0.1 ml), add sterile water to 1000 ml.

2.4.5. Tryptic Soy Broth (TSB)

1.7%(w/v) Digested Casein, 0.3%(w/v) Soybean Meal, 0.25%(w/v) Dextrose, 85.4mM NaCl, 0.15mM K_2HPO_4, pH 7.3. This medium was used for *S.aureus* cultures.

2.4.6. Brain Heart Infusion Broth (BHI)

0.6%(w/v) Brain Heart Infusion, 0.6%(w/v) Peptic Digest Animal Tissue, 85.4 mM NaCl, 0.3%(w/v) Dextrose, 1.45%(w/v) Pancreatic Digest of Gelatin, 18mM Na_2HPO4, pH7.4. The medium was used for the growth of *S.aureus* cultures.

2.4.7. Antibiotics

Ampicillin (dissolved in water) was used in both plate and broth cultures at a final concentration of 100 μg/ml. Kanamycin (dissolved in water) was in 50 μg/ml at final concentration. Chloramphenicol (dissolved in ethanol) was used at 170 μg/ml at final concentration. Tetracycline (dissolved in ethanol) was used at 50 μg/ml final concentration.

2.5. Bacteria and plasmids

2.5.1. Genotypes of *E. coli* strains and their usage

TOP 10 cell:F^-*mcr*AΔ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15Δ*LacX74recZ1deoRara*

D139 Δ(*ara-leu*)7697*gal*U*gal*K*rps*L(Str^R) *end*A1*nup*G. (Invitrogen). This strain was used for TOPO vector cloning.

DH5α: F?Φ80d/*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1*end*A1 *sd*R17(rk$^+$,mk$^+$) *pho*A *sup*E44λ$^-$*thi*-1*gyr*A96 *rel*A1. (GIBCO BRL). This strain was used for general cloning and plasmid DNA preparation.

BL21: F$^-$*ompT hsdS$_B$*(r$_B^-$m$_B^-$)*gal dcm.*(GIBCO BRL). This strain was used for general heterologous protein expression.

BL21 (DE3): F$^-$*ompT hsdS$_B$*(r$_B^-$m$_B^-$)*gal dcm*(DE3)(Novagen). From BL21 (DE3) to NovaBlue (DE3), these strains were used to express proteins, which were cloned into pET vectors.

BL21 (DE3)pLysS: F$^-$*ompT hsdS$_B$*(r$_B^-$m$_B^-$)*gal dcm*(DE3) pLysS (Novagen), protein expression strain.

BL21 (DE3) pLysE: F$^-$*ompT hsdS$_B$*(r$_B^-$m$_B^-$)*gal dcm*(DE3) pLysE (Novagen), protein expression strain.

AD494 (DE3): Δ*ara-leu*7967 Δ*lac*74 Δ*phoAPvuIIphoR* Δ*mal*F3F' [*lac*$^+$ (*lacI*^{q})*pro*] *trx*B::*kan* (DE3)(Novagen), protein expression strain.

AD494(DE3)pLysS:Δ*ara-leu*7967Δ*lac*74Δ*phoAPvuIIphoR* Δ*mal*F3F'[*lac*$^+$(*lacI* ^{q})*pro*] *trx*B::*kan* (DE3) pLysS (Novagen), protein expression strain.

HMS174 (DE3): F$^-$*rec*A1 *hsd*R(rk12$^-$mk12$^+$)R*if*^{R} (DE3), (Novagen), protein expression strain.

HMS 174 (DE3) pLysS: F$^-$*rec*A1 *hsd*R(rk12$^-$mk12$^+$) R*if*^{R} (DE3) pLysS, (Novagen), protein expression strain.

BLR (DE3) pLysS: F^-*ompT* *hsd*$S_B(r_B^- m_B^-)$ *gal dcm* Δ(*sri-recA*)306::Tn 10 (DE3) pLysS, (Novagen), protein expression strain.

NovaBlue (DE3): *endA1 hsdR17*($r_{K12}^- m_{K12}^+$) *supE*44 *thi*-1 *recA*1*gyrA*96 *relA*1 *lac*[F'*proA*$^+$ *B*$^+$ *lacI*^{q} ZΔM15::Tn10](DE3)(Novagen).

2.5.2. *B. subtilis* strain 168: BD170

This strain was a gift from Dr. Neil Fairweather (Imperial College of Science, Technology and Medicine, London).

2.5.3. *S.aureus*

The *S.aureus* isolates used in this thesis were purchased from the Public Health Laboratory Service (PHLS; London). The strains 8325 PS47 (36) & 8409 * 47 possess no resistance to any antibiotics.

2.5.4. Plasmid vectors

TOPO PCR 2.0 vector (Invitrogen). This vector was used for cloning PCR products and generating plasmid DNA for sequencing. It possesses ampicillin and kanamycin resistance genes and a *lacZ* operator in frame with a multi-cloning site for screening. The vector has been engineered to contain an extra T-residue at each 3'-DNA end. The Topoisomerase is used to ligate linear PCR fragments that are generated with an additional A-residue incorporated at their 5'-ends by DNA polymerase in PCR.

The vectors pET 11a, pET15b, pET21b, pET28a (Novagen), are used for the construction of intracellular fusion protein expression. These factors are used to introduce an N- or C-terminal polyhistidine tag (histag) on the heterologous protein. This set of vectors possesses a T7 promoter and T7 terminator at both ends of a multi-cloning site.

The vector pCWori was a gift from Dr. Ignacio Rodriguez-Crespo, (University of California, USA). The vector has been used successfully for the expression of the N-terminal domain of mammalian NOS enzymes (Rodriguez-Crespo, I., *et al.*, 1996; Wu, C., *et al.*, 1996; Roman, K.M., *et al.*, 1998; etc), and for the expression of the *E. coli CheW* protein (Liu, J., 1989). pCWori possesses a double *tac* promoter, and *lacZ'* and *lacI*^{q} genes. An M13 origin of replication is included for generating single-stranded DNA. In addition, the vector has a ColE1 origin of replication. For expression of NOS-like proteins, the *CheW* DNA fragment is removed by restriction enzyme digestion with *NdeI/XbaI*. The cassette expressing the target gene (bacterial NOS-domain) is inserted into the same sites.

pACYC184 (New England BioLabs) is a low copy number cloning vector. The vector has a p15A origin of replication and carries two antibiotic resistance genes (*tet* and *cat*). The vector was used for the co-expression of two proteins in one *E. coli* background in order to overcome problems with plasmid incompatibility.

The pBC SK(-) phagemid vector (Stratagene) was used for general cloning purposes. It contains an extensive polylinker flanked by promoter sequences for T_3 and T_7 RNA polymerase along with a *lacZ* gene within the multi cloning sites. Multi-cloning sites are located proximal to the N-terminal coding region of *lacZ* gene for selection purposes. This vector was used as a starting point for the construction of allelic replacement vectors for SANOS deletion experiments in *S.aureus*.

The plasmid pProEX HT (GIBCO BRL) is a fusion expression vector used in *E. coli*. It contains a 6-histidine sequence at the N-terminal region proximal to a multi-cloning site. The vector contains a synthetic *trc* promoter and a *lacI*^{q} operator for inducible expression. There is an rTEV protease cleavage site between the 6 histidines and the cloning sites allowing the extra 6 histidines to be removed from any recombinant protein by proteolytic cleavage.

pTrc99A (Pharmacia Biotech) is a controlled high-level expression vector. It has a synthetic *trc* promoter and a *lacI*^{q} operator and includes a multi-cloning site and

ampicillin resistance gene.

pGEX4T-2 (Pharmacia Biotech). The GST-fusion vectors are designed for inducible, high-level intracellular expression of genes or gene fragments as fusion with *Schistosoma japonicum* glutathione S-transfease (GST). Fusion proteins were purified from bacterial lysates by affinity chromatography using glutathione-Sepharose. PGEX 4T-2 contains a *tac* promoter, an internal *lacI*^{q} gene (for use in any *E. coli* host) and a thrombin protease recognition site for cleavage of the desired protein from GST.

pT181 is a *S.aureus* cloning vector and was from Dr. Michael Lockyer, (Arrow Therapeutics Limited). pT181 occurs naturally in *S.aureus* and encodes inducible resistance to tetracycline (Projan, S.J., *et al.*, 1985). The tetracycline gene and its control region were used for cloning fragments for allelic replacement in *S.aureus.*

pCWGST is a fusion expression vector constructed for this study. The vector is based on pCWori. The GST and multi cloning site fragment was amplified by PCR from pGEX4T-2 with the restriction enzyme sites *NdeI/XbaI* at either end. The PCR product was cloned into pCWori double digested with *NdeI/XbaI*, and the junctions verified by DNA sequencing. The vector retains not only the characteristics of pCWori, but also includes more cloning sites and can be used to generate GST fusion proteins.

2.6. Antibodies

2.6.1. Antihistag antibody

Monoclonal anti-polyhistidine peroxidase conjugate: clone His-1 purified mouse immunoglobulin was from Sigma.

2.6.2. Anti rabbit IgG antibody

Anti-rabbit IgG horseradish peroxidase conjugate was from Transduction Labs.

2.6.3. Anti *yflM* antiserum

The oligopeptides used to rise anti serum from rabbit were:

SCSLTAACEELGWRGERTDF (*yflM*121) and

HTAASQFKRFEEQEEEAGRK (*yflM*276)

The anti-serum was raised by Regal Rabbits PLC from 4 rabbits.

2.7. DNA manipulations

2.7.1. Restriction enzyme digestion

Restriction digests were performed in accordance with manufacturer's instructions. The amount of DNA to be digested was between 0.5-3 μg. Typically, digestion conditions involved incubating for 1 hour at 37°C in presence of 5-10 units of restriction enzyme.

2.7.2. Dephosphorylation of DNA ends

To avoid self-ligation of vector sequences, DNA digestion was followed by adding calf intestinal alkaline phosphatase (CIAP) in CIAP buffer. The amount of the enzyme was kept in appropriate concentration in accordance with the manufacturers guidance. The reaction was carried out for 15 minutes at 37°C.

2.7.3. Blunt-ending of DNA 5' overhangs

DNA fragments (2.7.6) were blunt-ended by treatment with DNA polymerase I Klenow fragment. The reaction was performed by adding dNTP to 1 mM and 1x Klenow buffer in accordance with the manufacturers recommendations for 30 minutes

at 37°C.

2.7.4. Phenol chloroform extraction and ethanol precipitation

Phenol was buffered by Tris buffer (GIBCO BRL). An equal volume of phenol/ chloroform/Isoamyl alcohol (25:24:1) was added to the aqueous phase containing the DNA to be extracted. After a brief vortexing the layers were separated by centrifugation (13,000 xg for 10 minutes at 4°C in a bench-top microfuge). The DNA was precipitated by adding 0.1 volumes of 3M sodium acetate (pH5.2) and 2.5 volumes of ethanol. The sample was incubated for 30 minutes on ice. DNA pellets were washed by 70% ethanol, and recovered by centrifugation (13,000 xg, 15 minutes at 4°C) and re-suspended in water or appropriate buffer. Samples prepared in this way were substrates for PCR templates or restriction enzyme digestion.

2.7.5. Non-denaturing agarose gel electrophoresis

The DNA samples were loaded into 0.6 to 1.5% (w/v) agarose gels containing 0.1 μg/ml ethidium bromide made in 0.5×TBE buffer (GIBCO BRL), mixed with 0.2 volume of 5× loading buffer provided by GIBCO BRL (50% glycerol, 5×TBE, 0.1%(w/v) bromophenol blue). Gels were run in 0.5×TBE buffer at lower than 5 volts/cm gel length under constant voltage. Gels were examined and photographed under UV using an Ultraviolet Transilluminator (PLS) and Digital Graphic Printer (SONY).

2.7.6. Recovery of DNA fragments from agarose gels

Non-denatured agarose gels were used for the recovery of DNA fragments produced by restriction enzyme digestion or PCR. Gels were examined under UV light first to ensure that the given fraction was separated by electrophoresis. The required band was cut out using a disposable blade. Minimum UV exposure time was used to avoid DNA damage. The gel slice was transferred into a 1.5 ml eppendorf tube and DNA was extracted by using a QIAquick Gel Extraction kit according to the manufacturers

instructions. DNA recovery was examined by electrophoresis in agarose gels with an aliquot of the obtained sample (2.7.5). The recovered DNA fragment could be used as a PCR template, for ligations, probe labelling, or for DNA sequencing.

2.7.7. Ligation of DNA fragments

The DNA fragments used in ligation reactions were produced by non-denatured agarose electrophoresis (2.7.5) and recovered from the gel (2.7.6).

DNA fractions to be ligated were added together into an eppendorf tube to a final volume 8 μl with sterile water. Usually the insert DNA was added in a 5-10 fold molar excess over the vector DNA. 1 μl of T_4 ligase and 1 μl 10×T_4 ligase buffer (200 mM Tris.HCl pH7.6, 50 mM $MgCl_2$, 50 mM DTT, 50 μg/ml BSA, 10 mM ATP) were added into the tube containing DNA fragments. The reaction was carried out overnight at 16°C. The ligase was inactive by heat for 10 minutes at 70°C. One fifth of the ligation mixture was used for bacterial transformation (2.7.8).

2.7.8. Transformation of *E. coli* with plasmids or ligation mixtures using chemical-competent cells

The process to make chemical competent cells is described as follows: A colony of a particular strain of *E. coli* was inoculated into 5 ml LB and grown overnight at 37°C with vigorous shaking. The overnight culture was diluted 100-fold to the appropriate volume with fresh LB and grown for another 2-3 hours until the OD_{600} measurement reached 0.2-0.4. The culture was chilled on ice for 30 minutes and the cell pellet was obtained by centrifugation in 4,000 xg for 10 minutes at 4°C. The pellet was re-suspended in ice cold 50 mM $CaCl_2$ in 0.5x volume of the original culture and incubated for another 30 minutes on ice. The cells were recovered by centrifugation in 4, 000 xg for 10 minutes at 4°C. The cells were re-suspended in 0.1 volumes of the original culture in ice-cold 50 mM $CaCl_2$ for 10 minutes. 0.1 ml aliquots of the cells were transferred into sterile eppendorf tubes on ice. 2 μl of ligation mix or approximately 1 ng of uncut plasmid DNA was added to the cell aliquot followed by

gentle mixing and incubating on ice for 30 minutes. The cells were heat shocked at 42°C for 40 seconds, and then transferred to on ice for 2 minutes. 0.5 ml SOB medium (GIBCO BRL) was added into the tubes and the bacteria grown for 30 to 60 minutes at 37°C. One-fifth of the culture was spread onto LB-agar plates containing appropriate antibiotics with an 'L-shaped' sterilised disposable spreader. The plates were incubated inverted overnight at 37°C.

2.7.9. Mini-plasmid purification

A colony of *E. coli* with the recombinant plasmid of interest was inoculated into 2 ml of sterile LB containing appropriate antibiotics, and grown overnight at 37°C with vigorous shaking. The cell of the culture was spun down at 10,000 xg for 1 min at room temperature. The medium was discarded and the cells were washed once with cold PBS.

Small-scale plasmid purification was carried out using the Wizard™ Miniprep DNA Purification System (Promega), according to the manufacturers instructions.

The purified plasmid DNA could be used for restriction enzyme digestion or subcloning.

2.7.10. Large-scale preparation of plasmid DNA

The method used was the alkaline lysis method as described by Sambrook, J., *et al.* (Sambrook, J., *et al.*, 1989). A single colony, containing the desired plasmid, was inoculated into 2 ml LB medium with appropriate antibiotics and grown 6-8 hours at 37°C with vigorous shaking. 1 ml of this culture was added into 100 ml of fresh medium, (pre-warmed to 37°C with appropriate antibiotics) and cultured overnight at 37°C. The cells were pelleted by centrifugation and the pellet was washed once in ice-cold PBS buffer. A QIAGEN Plasmid Maxi kit was used to purify plasmid DNA using the manufacturers instructions. The plasmid DNA was re-suspended in sterile water for further manipulations such as subcloning, DNA sequencing or probe

labelling.

2.7.11. Determination of DNA concentration

A spectrophotometer was used for quantitating the amount of DNA. Readings were taken at dual wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD unit corresponds to approximately 50 μg/ml for double-stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid (Sambrook, J., *et al.*, 1989).

2.7.12. PCR amplification and TOPO vector cloning

PCR is an effective method to amplify the target DNA sequence so as to generate large amounts of it for further manipulations. The PCR was performed using GeneAmp PCR reagents (System 2400 or 9600, Perkin Elmer). The reaction mixture contained 50 to 100 ng of template DNA, 10 pM each primer, 200 μM dNTP, 1×*Pfu* DNA polymerase buffer (20 mM Tris.HCl pH8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA) and 5 units of *Pfu* DNA polymerase in 100 μl reaction volume. Typical PCR was carried out for 30 cycles with each cycle comprising denaturation (30 seconds at 95°C), annealing (1 minute at 50°C) and extension (1 to 5 minutes at 72°C).

For the usage of the TOPO vector, 1 unit of *Taq* DNA polymerase was added into the PCR tube and the tube was incubated for 15 minutes at 72°C.

One particular feature used in the expression work was the design of specific primers. To aid in subsequent protein purification, a 6-histidine coding sequence was designed before the actual N-terminal methionine start codon. Some primers were designed to allow the incorporation of a 6-histidine tag at the C-terminus.

PCR®2.1-TOPO vector was used to clone PCR products (Invitrogen). The cloning process was guided by the producer's instructions. 100 ng of recovered DNA fragment (2.7.6) was mixed with 1 µl of TOPO vector to a final volume of 5 µl with sterile water in an eppendorf tube. For ligation, the tube was incubated for 5 minutes at room temperature. One vial of TOPO 10 cells (Invitrogen) was added to the mix with 2 µl 0.5 M β-mercaptoethanol. 2 µl of the ligation mixture was subsequently added in the tube. The mixture was incubated for 30 minutes on ice, and transformed cells were heat shocked for 30 seconds at 42°C and put on ice for 2 minutes. 0.2 ml of SOC (GIBCO BRL) medium was added, and the tube was incubated for 30 minutes at 37°C. A sample of 0.1 ml of the transformation reaction was poured onto an LB agar plate (supplemented with 100 µg/ml ampicillin). The plate was incubated inverted overnight at 37°C.

2.7.13. Double-stranded DNA plasmid sequencing

The DNA sequence of the gene encoding SANOS was generated on an Applied BioSystems DNA Sequencer at GlaxoWellcome, Medicines Research Centre, Stevenage. The DNA sequence of the gene encoding STAPHRED was generated on a CEQ 2000 DNA sequencing system at the Wolfson Institute for Biomedical Research. The sequence of *yflM* was verified manually.

For double-stranded DNA sequencing, 2.5 µg plasmid DNA was denatured by 0.2 M NaOH in a 10 µl volume at 37°C for 15 minutes. The primer was added to the mixture to a final concentration of 0.35 ng/µl. After briefly vortexing, DNA was recovered by centrifugation in a bench-top centrifuge at 13,000 xg for 10 minutes at 4°C following the addition of 3 µl of 3M potassium acetate pH 5.2 and 75 µl 100% ethanol. The DNA pellet was washed in 100 µl 70% ethanol, recovered by centrifugation and dried under vacuum for 15 minutes. The DNA and primer mixture was re-suspended in 8 µl sterile water. 2 µl of USB reaction Buffer (Amersham) were added. The labelling reaction was carried out in a 0.5 ml eppendorf tube as follows: 10 µl of the DNA/primer, 1 µl of 0.1M DTT, 2 µl 1:5 diluted labelling Mix, 0.5 µl [α-

^{35}S] dATP and 2 µl of 1:8 diluted T7 sequenase v2.0. The tube was incubated for 3 minutes at room temperature. At the same time, a microtitre plate was incubated at 37°C with 2.5 µl each of the dideoxynucleotides ddATP, ddCTP, ddGTP and ddTTP in separate wells. 3.5 µl labelling mix was added into each well. The plate was incubated for 3 minutes at 37°C. The sequencing reaction was stopped by adding 4 µl of stop solution to each well of the plate. When the sequencing gel was ready for loading, the microtitre plate containing the sequencing reactions was heated at 75°C for 3 minutes and immediately transferred onto ice. 2.5 µl of the sequencing reaction mix was loaded to each line of the polyacryamide/urea DNA sequencing gel according to the given loading order.

The electrophoresis was performed at 1350 volts (constant voltage) for 2-3 hours. The gel was transferred onto a strip of Whatman 3MM filter paper, covered by cling film and dried at 80°C for 1 hour.

The sequencing results were obtained by exposing X-ray film to the dried gel in a cassette. The film was generally developed after overnight exposure at -20°C.

2.7.14. DNA probe labelling

Hybridisation probes were generally derived from PCR products. They were labelled using a Random Primed DNA Labelling kit (Roche). The process was performed according to the manufacturer's instruction.

2.8. RNA manipulation

2.8.1. RNA extraction

Cultures of *S.aureus*, *B. subtilis* and *E. coli* were used for RNA extraction. The cells were recovered by centrifugation and re-suspended in 0.4 ml TE buffer in a 1.5 ml eppendorf tube. RNA was extracted by the addition of 0.5 ml phenol:chloroform: isoamyl alcohol (25:24:1) (GIBCO BRL). The layers were separated by

centrifugation at 13,000 xg at 4 °C for 10 minutes. The phenol extraction procedure was repeated twice, and RNA was precipitated by adding 0.1 volume of 3 M sodium acetate pH 4.2 and 3 volume of ethanol followed by centrifugation at 13,000 xg for 15 minutes at 4°C. The RNA pellet was re-suspended in a small volume of sterile water, and mixed with 3 volumes of 4×loading buffer. The sample was incubated for 15 minutes at 65°C prior to separation by denaturing agarose gel electrophoresis.

For some experiments, RNA was also extracted by RNeasy Mini kits (Qiagen). The process was guided by the producers instructions.

2.8.2. Denaturing agarose gel electrophoresis

Denaturing agarose gel electrophoresis was performed according to the instruction of the NorthernMax™ Northern Blotting Kit (Ambion). 1 gram of the agarose was melted in 90 ml of sterile DEPC-water. 10 ml of 10×Denaturing Gel buffer was added. The gel was set in a fume hood. The electrophoresis was run at 5volts/cm gel (constant voltage).

2.8.3. Northern blot and hybridisation

The separated RNA was blotted onto a Hybond-N Nylon membrane (Amersham) and cross-linked onto it by baking at 80°C for 15 minutes. Pre-hybridisation, hybridisation, washing, and exposure were carried out according to the manufacturer's instruction (Ambion). The film was developed on a Fully Automatic X-ray Film Processor(X-ograph Imaging System Compact X4, UK).

2.9. Protein analysis

2.9.1. SDS-PAGE

Proteins were separated by SDS-PAGE in Mini-PROTEAN II Cells (BioRad). The gels were composed of two parts. The lower part, the separating gel, was poured first

and contained: 10-15%(w/v) of acrylamide (37.5:1 bis, Bio Rad), 0.3M Tris.HCl pH8.8, 0.1%(w/v) SDS, 0.1%(w/v) ammonium persulfate, and 0.04%(v/v) TEMED. The top of the gel was covered by butanol until it was set. After setting, the butanol was removed and the gel washed with water. The upper part of the gel, stacking gel, was poured, comprising: 4%(w/v) acrylamide (37.5:1 bis-acryamide), 0.125 M Tris.HCl pH6.8, 0.1%(w/v) SDS, 1%(w/v) ammonium persulfate, 0.1%(v/v) TEMED. Protein samples were mixed with an equal volume of 2× SDS loading buffer (0.125 M Tris-HCl pH 6.8, 20%(v/v) glycerol, 4 %(w/v) SDS, 10%(v/v) 2-mecaptoethanol, 0.002% (w/v) bromophenol blue), heated at 100°C for 5 minutes, and loaded onto the gel. The electrophoresis was run for between 1-1.5 hours under constant current (40 milliamps) in a buffer containing: 50 mM Tris.HCl pH 8.3, 250mM glycine, 0.1%(w/v) SDS.

2.9.2. Coomassie blue staining of SDS-PAGE gels

The gel was removed from gel box set following electrophoresis (2.9.1) and put into 5 volume of stain buffer comprising 0.25% Coomassie brilliant blue, R-250, solution [5%(v/v) methanol, 10%(v/v) acetic acid] for 1 hour in a low speed shaker. The gel was de-stained by incubation in de-staining buffer containing 5%(v/v) methanol and 10%(v/v) acetic acid with several changes. Gels were dried using the Promega gel drying kit according to the manufacturers instruction.

2.9.3. Western blot and immunological detection

Proteins separated by SDS polyacrylamide gel electrophoresis were electrophoretically transferred onto nitrocellulose membrane. The gel was soaked in blotting buffer (20 mM Tris.HCL pH8.0, 150 mM glycine, 20%(v/v) methanol) for 10 minutes. A sandwich of 3 sheets of filter paper (Whatman 3MM) followed by a sheet of membrane, the gel, and 3 sheets of 3MM were assembled. All the above material was pre-wetted in blotting buffer. The sandwich was submerged into blotting buffer, in an electro-blotting tank with the membrane between the gel and the positive electrode. The gel was blotted at 150 mA for 1 hour.

The membrane containing the transferred protein was blocked with 5%(w/v) dried milk powder in PBST [0.1%(v/v) Tween-20 in PBS] at 4°C overnight. The primary antibody (concentration as recommended by the producers) was added into the blot and incubated for 1 hour at room temperature in 5%(w/v) milk powder/PBST. The membrane was washed 3×15 minutes with PBST.

The secondary antibody was added in appropriate dilution and incubated with the membrane for another hour in the same buffer (the secondary antibody was peroxidase-conjugated antibody). The membrane was washed 3×15 minutes and 3×5 minutes with PBST. The bound immuno-complexes were detected by the enhanced chemiluminescence (ECL) method, or by DAKO liquid DAB kit, guided by the producer's instruction.

2.9.4. Determination of protein concentration

The Bradford protein assay (BioRad) was used to determine the protein concentration. The concentration was measured according to the instructions with BSA as the standard. 1 μl of protein sample was added into a 1 ml cuvette with 0.8 ml sterile water, followed by adding 0.2 ml BioRad Protein Assay Reagent. The absorbance at 595 nm was recorded after the cuvette had been mixed well. The BSA fragment V was used to generate a standard curve.

2.9.5. Expression of histidine-tagged (his-tag) fusion proteins

Two kinds of his-tagged protein were expressed. One was protein expressed in the commercially available pET or pProEX HT vectors, which possesses his-tags on the C- or N-termini. The other class was protein with a his-tag introduced by PCR at the C- or N-termini.

A single colony was inoculated into 5 ml Terrific broth with appropriate antibiotics and grown in a 37°C shaker overnight. For a time-course to follow the induction of protein expression, the overnight culture was diluted 100-fold in 10 ml Terrific broth

with appropriate antibiotics, and incubated at 37°C shaker until the OD_{600} reached 0.8-1.0. The induction was observed at different times. IPTG was used for induction (1 mM), for heme proteins, δ-aminolevulinic acid was added (0.45 mM) with growth at 23°C. Aliquot of the time-course was recovered by centrifugation. The final volume of the re-suspended cells was adjusted to equal OD unit/ml by adding appropriate SDS-PAGE loading buffer (0.1 M Tris.HCl pH6.8, 10%(v/v) glycerol, 1%(w/v) SDS, 0.002%(w/v) bromophenol blue) for analysis.

For purification of recombinant proteins, the overnight culture (30 ml) was diluted 100-fold into 3 litres of Terrific broth with appropriate antibiotics in 6×2.8 litre flasks. The induction of the culture was carried out until the OD_{600} reached 0.8-1.0 by adding IPTG to 0.5 mM (for heme proteins, δ-aminolevulinic acid was added to 0.45 mM) for 48 hours at 23°C. For the expression of recombinant STAPHRED the same conditions were used, except the temperature was 37°C for 3 hours. The cells were recovered by centrifugation for 15 minutes at 4°C at 4,000 rpm. The cell pellet was chilled on dry ice and stored at -80° until protein purification was carried out.

2.9.6. Expression of GST fusion proteins

pGEX4T-2 or pCWGST fusion protein was expressed in the BL21 strain of *E. coli*, which lacks an outer membrane protease (ompT). The fresh plasmid was transformed into BL21 cells. A single colony was inoculated into 10 ml and grown overnight at 37°C. Other processes are described in 2.8.6.

2.9.7. Purification of his-tag fusion proteins

The cell pellet containing recombinant his-tagged protein was re-suspended in buffer A (40 mM EPPS pH7.6, 250mM NaCl, 10%(v/v) glycerol, 3 mM DTT, 50 μg/ml lysozyme, 0.5 μg/ml each of PMSF, leupeptin and pepstatin A). For heme protein(s), 1 mM L- arginine was also added. Cells were resuspended at a ratio of 2 grams wet cell per millilitre of buffer. Sonication was used to disrupt cells using three 30-seconds pulses comprising 5 seconds burst/5 seconds rest. The cell debris was

removed by centrifugation at 13,000 xg at 4°C for 15 minutes. The supernatant was obtained by a further 1 h centrifugation at 100,000 xg at 4°C. TALON metal affinity resin was washed with buffer A in a 2cm/15cm column for chromatography. The supernatant was loaded into the column by a pump at a rate of 0.5 ml/minute. The column was washed with buffer A (in 8 to 10 column volumes) and with buffer A containing 10 mM imidazole in 5 column volumes. The protein was eluted from the column by 150 mM imidazole in buffer A. The peak aliquots were collected and pooled together. The OD_{280} was monitored during the whole process.

2.9.8. Ammonium sulphate precipitation

Ammonium sulphate precipitation is a common method to concentrate protein in solution. In this study, the solid ammonium sulphate was added into the protein solution which eluted from the TALON column to 55%(w/v) of the final volume. After stirring for 20 minutes at 4°C, the precipitated protein was recovered by centrifugation at 15,000 xg at 4°C for 50 minutes. Buffer A was used to dissolve the protein in the minimum volume. The protein then was then dialysed overnight at 4°C against buffer A. The dialysed protein was aliquoted (0.2 ml) into 1.5 ml tubes pre-cooled in dry ice. Samples were stored at -80°C until needed.

2.9.9. Urea denatured purification of his-tagged protein from *E. coli*

The his-tagged protein was expressed as described in 2.9.5. The cell pellet was thawed and resuspended by in 0.5 ml buffer B (8M urea, 0.1 M NaH_2PO_4, 0.01 M Tris.HCl pH8.0). The mixture was incubated for 60 minutes at room temperature. The supernatant was separated by centrifugation at 13,000 xg at 4°C for 30 minutes. 1 ml Ni-NTA slurry was added to 4 ml of the supernatant, and mixed by rotating at room temperature for 60 minutes. The mixture was loaded onto an empty column. The column was washed two times with 4 ml buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris.HCl pH6.3). The protein was eluted by 4×0.5 ml washes of buffer D (8M urea, 0.1 M NaH_2PO_4, 0.01 M Tris.HCl pH5.9), followed by 4×0.5 ml of buffer E (8M

urea, 0.1 M NaH_2PO_4, 0.01 M Tris.HCl pH 4.5). The eluted protein was used as a molecular marker in SDS-PAGE.

2.9.10. Antibody IgG isolation by FPLC

The antiserum was loaded onto an ANX column in the FPLC UPC-900/Frac-950 system. After washing by PBS buffer, IgG was eluted by PBS with 0.5 M NaCl. The pH was adjusted to 7.0 by 1 M Tris.HCl, pH12.

2.10 NOS heme domain functional assay

2.10.1. Heme determination

Heme determination was carried out according to Klatt *et al.* (1996). A solution of NOS, or bacterial NOS-like domain (1 mg/ml) was thoroughly mixed with an equal volume of freshly prepared reagent A (0.2 ml of 5 M NaOH, 2.5 ml pyridine, and H_2O to 5 ml), followed by the immediate addition of a few grains of solid sodium dithionite and mixing. The spectrum of the reduced pyridine hemochrome was recorded in a 0.5 ml cuvette from 370 to 700 nm against blanks containing buffer and reagent A. Heme concentrations were calculated from the differences in absorbance at 556 versus 540 nm using an absorbance coefficient 0f 22.1 $mM^{-1}cm^{-1}$.

2.10.2. Spectrophotometry of compounds binding

Inhibitor, substrate and co-factor binding experiments were carried out on N-terminal NOS-domains and on bacterial NOS-like protein samples. A Perkin-Elmer Lambda 7 UV/VIS spectrophotometer and a Shimadzu UV-2401 PC, UV-VIS recording spectrophotometer, were used.

Samples were reacted in a cuvette following a modification of the McMillan protocol (1993). 1 ml of PBS, 3-5 μg of protein sample, 100 μM DTT and 100 μM BH_4 were mixed. The scan range was from 300 nm to 700 nm against water and the changes of

absorption in 396 nm were recorded for calculation. Imidazole-shifting experiments were carried out first. Binding tests for other compounds were performed in the same solution as described above plus 350 mM imidazole.

2.10.3. H_2O_2 shunt

The method was modified from Ghosh *et al.* (Ghosh, D.K., *et al.*, 1997). The reaction was performed in an eppendorf tube containing: 50 mM HEPES, 500 μM DTT, 10 units SOD, 0.5 mg/ml BSA, 150 nM NOS heme domain protein, 100 μM BH_4. The tube was incubated for 10 minutes at 25°C. Subsequently the NOS inhibitors and NOHA and 300 mM H_2O_2 were added to start the reaction. The tube was incubated for another 30 minutes at 25°C.

0.5 ml Greiss reagent A (1g sulphanilamide in 2.94 ml concentrated phosphoric acid, made up to 100 ml with distilled water) was added into 0.5 ml of the reaction and held on ice for 10 minutes. Then 0.5 ml Greiss reagent B was added (0.5 g NEDA in 2.94 ml concentrated phosphoric acid made up to 100 ml with distilled water). The absorbance at 548 nm was recorded after incubating for each 10 minutes time at room temperature.

2.11 Finding NOS-like sequences in bacteria

2.11.1. *yflM* in *B.subtils*

The whole genomic DNA sequence of *B. subtilis* was published in 1997 by Kunst *et al.* (Kunst, F., *et al.*, 1997). Bioinformatic assessement suggested that the organism contains 4,100 protein-coding genes, and a protein encoded by the *yflM* gene was noted as having sequence homology to nitric oxide synthase in mammals.

2.11.2 BLAST searching for NOS-like sequences in bacteria

Using the human iNOS protein sequence as an inquiry template, a NOS heme-domain like protein and a NOS reductase-domain like protein were found in the genome of *S.aureus* (unfinished microbial genomes, TIGR). These two proteins have been called SANOS and STAPHRED respectively.

2.11.3 Protein searches

The searching was performed using the GCG Package, Human Genome Mapping Project Resource Centre, UK. The charges and isoelectric points of SANOS and STAPHRED were also calculated by the GCG package.

2.11.4 Peptide alignment

The proteins were aligned using the ClustalW programme in the EMBL Outstation, European Bioinformatics Institute, UK; and BioEdit Sequence Alignment Editor software, USA. The identity and similarity of SANOS and STAPHRED with other NOS proteins were calculated using the above database and software.

CHAPTER 3

CLONING AND EXPRESSION OF *yflM*, A GENE ENCODING A NOS-LIKE PROTEIN FROM *B. subtilis*

CONTENTS

3.1. Introduction

B. subtilis is an aerobic, endospore-forming, Gram-positive, rod-shaped bacterium commonly found in soil, water sources and in association with plants. *B. subtilis* and its close relatives are an important source of industrial enzymes (such as amylase and proteases), and much of the commercial interest in these bacteria arises from their capacity to secrete these enzymes at gram per litre concentrations (Harwood, C.R., *et al.*, 1992).

The whole genomic DNA sequence of *B. subtilis* was finished in 1997 (Kunst, F., *et al.*, 1997). Its genome of 4,214,810 base pairs comprises 4,100 protein-coding genes. In the initial genome annotation, one of the proteins encoded by the *yflM* gene was noted as having similarity to mammalian NOS labelled as nitric oxide synthase.

yflM was the first gene encoding a bacterial NOS-like proteins studied in this project.

3.2. *yflM* in *B. subtilis*

According to the published *B. subtilis* genomic DNA sequence, *yflM* is one of the single copy genes. It has been classified as having homology to NOS, and has been placed in the 'detoxification' classification group (Kunst, F., *et al.*, 1997). A '*y*' prefix with the name indicates a gene of unknown function.

Appendix 1 shows the result of a BLAST search at the National Centre for Biotechnology Information (NCBI) site, U.S.A. *yflM* comprises 1011 base pairs and is capable of encoding a protein of 336 amino acids. The G+C ratio of *yflM* is 50.3%, and this compares to the whole genomic average of 43.5% in *B. subtilis*.

3.3. Alignment of *yflM*, SANOS, and NOS

When the *yflM*-encoded protein is aligned with SANOS (a NOS-like protein from *S.aureus* described in Chapter 4 and 5; **Figure 3.1**), the similarity is around 80%.

This suggests that the two proteins may possess similar properties. **Figure 3.2** shows the alignment of the *yflM*-encoded protein and human iNOS. Both the *yflM*-encoded protein and SANOS are similar in size, and can be aligned around the same region with respect to the human iNOS sequence. This alignment suggests that the *yflM*-encoded protein may not possess a BH_4 binding site, or a site for calmodulin binding. However, heme binding site alignments (**Figure 3.3**) suggest that the *yflM*-encoded protein and SANOS may be able to bind a heme group.

3.4. PCR amplification, molecular cloning and DNA sequencing

Based on the DNA sequence of *yflM* from Genebank, a pair of primers were synthesised to PCR amplify *yflM* from samples of *B. subtilis* genomic DNA. The *B. subtilis* used in these experiments was a gift from Dr. Neil Fairweather (Imperial College of Science, Technology and Medicine, London). Bacteria were grown on L-agar plates without any antibiotics at 37°C overnight. One colony was inoculated into a 150 ml flask containing 10 ml LB and grown overnight at 37°C with vigorous shaking. The DNA extraction was carried out as described in 2.7.4. DNA was kept at 0.1 mg/ml concentration at -20°C until needed.

The sequences of the primers are:

1) 5' primer 5'-GGGAATTCCATATGAAAGACCGTCTCGCG-3'
2) 3' primer 5'-GCGGGATCCTTACTCATAAGGCTTATCTTG-3'

A *BamHI* and an *XbaI* restriction enzyme site were designed into N- and C-termini of the primers to facilitate cloning in pET28a. The PCR reactions and TOPO vector cloning were performed as described in 2.7.12. **Figure 3.4** shows the PCR product of *yflM* amplification. *yflM* DNA sequence was verified according to the method described in 2.7.13. **Figure 3.5** shows part of *yflM* sequencing result.

Figure 3.1 Alignment of the *yflM*-encoded protein and SANOS

```
yflM  ----------------------MKDRLADIKSEIDLTGSYVHTKEELEHGAKMAWRNSN 37
SANOS MLFKEAQAFIENMYKECHYETQIINKRLHDIELEIKETGTYTHTEEELIYGAKMAWRNSN 60
                           ::.** **: **. **:*.**:*** :**********

yflM  RCIGRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPTITIFPPEEKGEKQVE 97
SANOS RCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPYITIYAPKD-GPK--- 116
      ********:****** ***  : .. .:: :**  ***:**::* ***:.*:: * *

yflM  IWNHQLIRYAGYESDGERIGDPASCSLTAACEELGWRGERTDFDLLPLIFRMKGDEQPVW 157
SANOS IFNNQLIRYAGYDN----CGDPAEKEVTRLANHLGWKGKGTNFDVLPLIYQLP-NESVKF 171
      *:*:********:.     ****. .:*  .:.***:*: *:**:****:::  :*.  :

yflM  YELPRSLVIEVPITHPDIEAFSDLELKWYGVPIISDMKLEVGGIHYNAAPFNGWYMGTEI 217
SANOS YEYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGGIVYPTAPFNGWYMVTEI 231
      ** * **: **** * .   : .*:****.*****:*.*::*** * :******** ***

yflM  GARNLADEKRYDKLKKVASVIGIAADYNTDLWKDQALVELNKAVLHSYKKQGVSIVDHHT 277
SANOS GVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAVYHSFKKEGVSIVDHLT 291
      *.**: *: **: *:***..: : :  *..: **:****** ** **:**:******* *

yflM  AASQFKRFEEQEEEAGRKLTGDWTWLIPPISPAATHIFHRSYDNSIVKPNYFYQDK---- 333
SANOS AAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDNTVKDPNFFYKKKESNA 351
      **.**: **.:* : **::**.*:** **:**: *  :*:.***:: .**:**:.*

yflM  ---PYE- 336
SANOS NQCPFHH 358
         *:.
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Figure 3.2 Alignment of the *yflM*-encoded protein and human iNOS

```
yflM  ------------------------------------------------------------
HiNOS MACPWKFLFRVKSYQGDLKEEKDINNNVEKTPGAIPSPTTQDDPKSHKHQNGFPQFLTGT 60

yflM  ------------------------------------------------------------
HiNOS AQNVPESLDKLHVTPSTRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIMNSK 120

yflM  -------------------------------------MKDRLADIKSEIDLTGSYVHTK 22
HiNOS SLTRGPRDKPTPVEELLPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTL 180
                                            **  :..**: **:*  *

yflM  EELEHGAKMAWRNSNRCIGRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPT 82
HiNOS DELIFATKMAWRNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSA 240
      :** ..:******: *****: *..*:*:* *.  * .*: : : :**  ***.*:**.:

yflM  ITIFPPEEKGEKQVEIWNHQLIRYAGYES-DGERIGDPASCSLTAACEELGWRGERTDFD 141
HiNOS ITVFPQRNDGKHDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFD 300
      **:** .:.*::::..*** ********:  **   ****: .:*  * :***: .   **

yflM  LLPLIFRMKGDEQPVWYELPRSLVIEVPITHPDIEAFSDLELKWYGVPIISDMKLEVGGI 201
HiNOS VLPLVLQAHGQD-PEVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGL 359
      :***::: :*:: *  :*:* .**:**.: **. * *.:* ****.:* :::* *****:

yflM  HYNAAPFNGWYMGTEIGARNLADEKRYDKLKKVASVIGIAADYNTDLWKDQALVELNKAV 261
HiNOS EFPACPFNGWYMGTEIGVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAAV 419
      .: *.************.*:*.* :**: *::*.  :*:.:.  :.****:*:.*:* **

yflM  LHSYKKQGVSIVDHHTAASQFKRFEEQEEEAGRKLTGDWTWLIPPISPAATHIFHRSYDN 321
HiNOS LHSFQKQNVTIMDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLN 479
      ***::**.*:*:*****:..* :. ::* .*    ..** **:**:* : * :**:.  *

yflM  SIVKPNYFYQDKPYE---------------------------------------------- 336
HiNOS YVLSPFYYYQIEPWKTHIWQDEKLRPRRREIRFTVLVKAVFFASVLMRKVMASRVRATVL 539
       ::.* *:** :*::
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Figure 3.3 NOS heme domain alignment

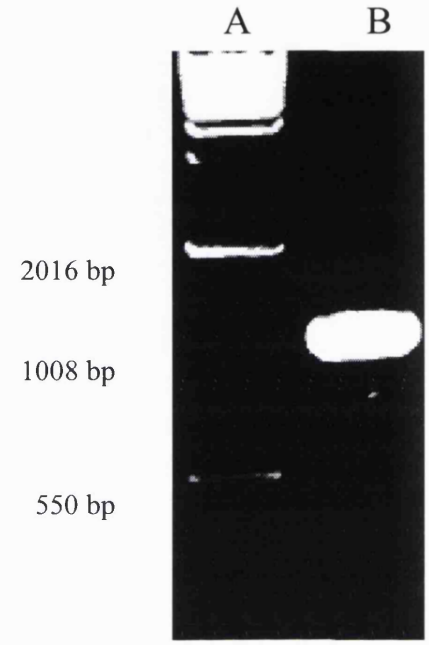
```
Murine iNOS          WRNAPRCIGRIQW
Rat nNOS             WRNAPRCIGRIQW
Guinea pig iNOS      WRNAPRCIGRIQW
Human iNOS           WRNAPRCIGRIQW
Dog iNOS             WRNAPRCIGRIQW
Chicken iNOS         WRNAPRCIGRIQW
Mouse nNOS           WRNASRCVGRIQW
Rabbit iNOS          WRNASRCVGRIQW
Bullfrog nNOS        WRNASRCVGRIQW
Human eNOS           WRNAPRCVGRIQW
Guinea pig eNOS      WRNAPRCVGRIQW
Dog nNOS             WRNAPRCVGRIQW
Pig iNOS             WRNAPRCVGRIQW
Bovine eNOS          WRNAPRCVGRIQW
Snail NOS            WRNAPGCIGRSQW
Drosophlia NOS       WRNSSRCIGRIQW
Mosquito NOS         WRNAPRCIGRIQW
Hornworm NOS         WRNATRCIGRIQW
SANOS (S. aureus)    WRNSNRCIGRLFW
YFLM (B. subtilis)   WRNSNRCIGRLFW
                     ***: **:** .*
```

***: designates identical residues;**

: : designates highly conserved residues;

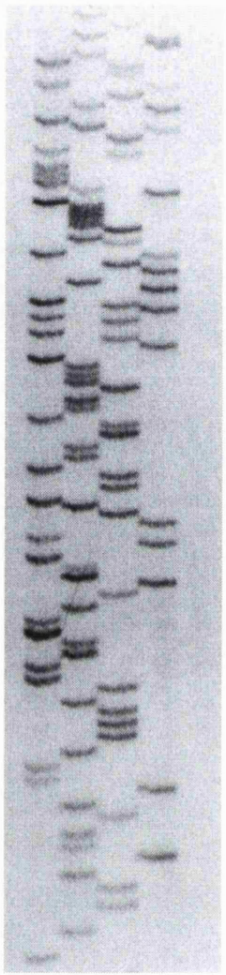
. : designates conserved residues.

Figure 3.4 PCR amplification of *yflM*



A. DNA marker. B. PCR product of *yflM*.

Figure 3.5 *yflM* DNA sequencing



The sequencing results of *yflM* are from last pair 727- 827 (from bottom to top). The sequence is:

GATTACAATACGGATTTATGGAAGGATCAAGCGC TAGTTGAATT GAATAAAGCTGTGCTGCACT CGTATAAAAAGCAGGGTGTCAGCA TCGTTGACCATCA

Figure 3.6 shows the *yflM* DNA and peptide sequences. These sequences are in 100% agreement with the sequences deposited in the NCBI database.

3.5. Expression of *yflM* in *E. coli*

After sequencing, *yflM* was cloned into the expression vector pET28a for expression in *E. coli* strain BL21 (DE3). Although different growth media, (for example Terrific broth, Luria-Bertani broth, 2×YT broth, and M9 minimum medium) were used for expression, no soluble protein could be detected.

Attempts were made to re-fold the insoluble pET28a-expressed *yflM*-encoded protein using 8M urea as a denaturant. Cells from a 100 ml induced culture of pET28a expressing *yflM* was pelleted by centrifugation (13,000 xg), and resuspended with 5 ml buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01M Tris.HCl pH 8.0). The sample was mixed gently for 60 minutes at room temperature and the cell debris pelletted by centrifugation for 30 minutes at 4°C at 13,000 xg. 1 ml of Ni-NTA resin slurry was added to the supernatant, mixing by rotating for 60 minutes at room temperature. The mixture was loaded into an empty column, and washed with 2×4 ml aliquots of buffer C (8M urea, 0.1 M NaH_2PO_4, 0.01M Tris.HCl pH6.3). The protein was eluted with 4×0.5ml buffer D (8M urea, 0.1M NaH_2PO_4, 0.01M Tris.HCl pH5.9), followed by 4×0.5 ml buffer E (8M urea, 0.1M NaH_2PO_4, 0.01 M Tris.HCl pH4.5).

Unfortunately, when denatured protein was renatured by decreasing the concentration of urea, the *yflM*-encoded protein invariably precipitated. Although this protein was of no further use for biochemical study, denatured purified *yflM*-encoded protein could be used as a protein marker in SDS-PAGE experiments to indicate the position of *yflM* encoded protein when SDS-PAGE was carried out.

A series of additional vectors were used to attempt to express soluble *yflM*-encoded protein in *E. coli*. These included pET21b, pET11a, pProxHT, pTrc99a, pGEX4T-2 and pCWori. The characteristics of the different vectors are shown in 2.5.4, and the data on the primers used for *yflM* expression are shown in **Appendix 5.** Expression

Figure 3.6 Deduced *yflM*-encoded protein sequence

```
GTG AAA GAC CGT CTC GCG GAC ATT AAA AGT GAA ATT GAC CTG ACC
 M   K   D   R   L   A   D   I   K   S   E   I   D   L   T    15

GGA AGC TAT GTA CAT ACG AAG GAA GAG CTG GAG CAC GGA GCG AAA
 G   S   Y   V   H   T   K   E   E   L   E   H   G   A   K    30

ATG GCT TGG AGA AAC AGC AAC CGC TGC ATC GGC AGA TTG TTC TGG
 M   A   W   R   N   S   N   R   C   I   G   R   L   F   W    45

AAT TCG CTG AAT GTT ATC GAC AGA CGA GAC GTC CGG ACG AAG GAG
 N   S   L   N   V   I   D   R   R   D   V   R   T   K   E    60

GAA GTG CGT GAT GCC CTC TTT CAC CAT ATT GAA ACC GCC ACC AAT
 E   V   R   D   A   L   F   H   H   I   E   T   A   T   N    75

AAC GGG AAA ATC AGA CCG ACC ATT ACG ATT TTC CCT CCG GAA GAG
 N   G   K   I   R   P   T   I   T   I   F   P   P   E   E    90

AAG GGT GAA AAG CAA GTC GAG ATC TGG AAT CAT CAG CTG ATC CGG
 K   G   E   K   Q   V   E   I   W   N   H   Q   L   I   R   105

TAC GCT GGA TAT GAG TCA GAC GGA GAA AGA ATC GGC GAC CCG GCT
 Y   A   G   Y   E   S   D   G   E   R   I   G   D   P   A   120

TCC TGT TCC CTG ACA GCA GCC TGC GAA GAG CTC GGC TGG CGC GGA
 S   C   S   L   T   A   A   C   E   E   L   G   W   R   G   135

GAG CGA ACG GAT TTT GAC CTG CTG CCG CTC ATT TTT CGC ATG AAA
 E   R   T   D   F   D   L   L   P   L   I   F   R   M   K   150

GGG GAC GAG CAG CCT GTC TGG TAT GAG CTG CCG CGT TCA CTT GTG
 G   D   E   Q   P   V   W   Y   E   L   P   R   S   L   V   165

ATT GAG GTT CCA ATC ACA CAT CCG GAC ATC GAG GCG TTT TCT GAT
 I   E   V   P   I   T   H   P   D   I   E   A   F   S   D   180

TTG GAG CTG AAG TGG TAC GGC GTG CCT ATT ATT TCT GAT ATG AAG
 L   E   L   K   W   Y   G   V   P   I   I   S   D   M   K   195

CTT GAG GTC GGG GGC ATT CAT TAT AAT GCC GCG CCA TTT AAC GGC
 L   E   V   G   G   I   H   Y   N   A   A   P   F   N   G   210

TGG TAT ATG GGC ACG GAG ATC GGA GCG AGA AAC CTC GCA GAT GAA
 W   Y   M   G   T   E   I   G   A   R   N   L   A   D   E   225

AAG CGG TAC GAC AAG CTC AAA AAA GTA GCG TCC GTG ATC GGC ATC
 K   R   Y   D   K   L   K   K   V   A   S   V   I   G   I   240

GCC GCT GAT TAC AAT ACG GAT TTA TGG AAG GAT CAA GCG CTA GTT
 A   A   D   Y   N   T   D   L   W   K   D   Q   A   L   V   255

GAA TTG AAT AAA GCT GTG CTG CAC TCG TAT AAA AAG CAG GGT GTC
 E   L   N   K   A   V   L   H   S   Y   K   K   Q   G   V   270

AGC ATC GTT GAC CAT CAT ACA GCG GCA AGC CAG TTT AAA CGG TTT
 S   I   V   D   H   H   T   A   A   S   Q   F   K   R   F   285

GAA GAA CAG GAG GAA GAA GCG GGC AGA AAG CTG ACG GGG GAC TGG
 E   E   Q   E   E   E   A   G   R   K   L   T   G   D   W   300

ACG TGG CTG ATT CCG CCA ATT TCA CCC GCT GCC ACT CAT ATC TTC
 T   W   L   I   P   P   I   S   P   A   A   T   H   I   F   315

CAC CGC TCC TAT GAT AAC TCA ATC GTT AAG CCG AAC TAT TTT TAT
 H   R   S   Y   D   N   S   I   V   K   P   N   Y   F   Y   330

CAA GAT AAG CCT TAT GAG TAA
 Q   D   K   P   Y   E   *                                   336
```

was attempted in a series of different *E. coli* strains in order to attempt to generate soluble *yflM*-encoded protein including: BL21 (DE3), BL21(DE3)pLysS, BL21(DE3) pLysE, AD494(DE3), AD494(DE3) pLysS, HMS174(DE3), HMS 174(DE3) pLysS, BLR(DE3) pLysS, and NovaBlue (DE3). The genotypes of this panel of different *E. coli* strains are shown in 2.5.1. This extensive series of experiments was extremely disappointing, and none of the plasmid/strain combinations resulted in increased solubility of the *yflM*-encoded protein. **Figure 3.7** shows some the expression profiles of the *yflM*-encoded protein. Protein was found exclusively in the insoluble fraction of cell lysates.

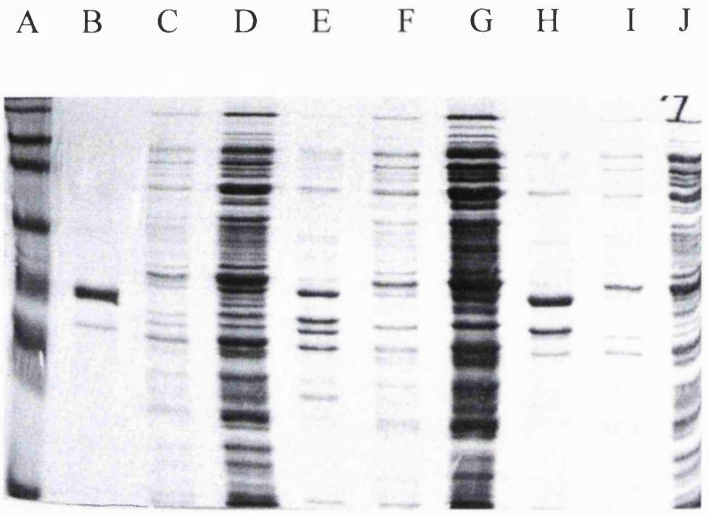
Detergents have been used to increase the solubility of recombinant expressed proteins, and experiments were carried out to assess their effect on the solubility of the *yflM*-encoded protein. A range of non-ionic, cat-ionic and zwitter ionic detergents were added to the purification buffer. The detergents tested were: NP-40 (0.05 to 0.3 mM); Chaps (0.2% to 0.6%); DDMAU (0.2% to 1 %); Triton-X100 (0.01% to 0.03%); n-Dodecyl β-D-Maltoside (0.1 to 0.6 mM) and n-Octyl-β-D-glucopyranoside (20 to 25 mM).

All these experiments failed to improve *yflM*-encoded protein solubility (data not shown).

To combine the properties of pCWori and pGEX-GST fusion expression, a fusion expression vector, pCWGST was constructed by introducing the GST-fusion element into the pCWori vector. **Figure 3.8** shows the construction of this novel vector (pCWGST), while **Figure 3.9** shows the resulting expression profile. The fusion protein *yflM*-GST was still retained in the pellet fraction following expression.

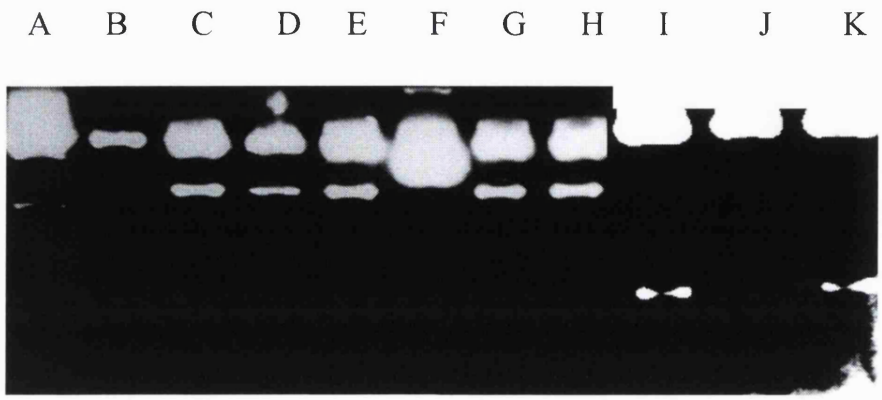
In conclusion, while *yflM* was expressed at high levels in *E. coli* to give a protein of the expected size, all attempts to generate soluble protein failed.

Figure 3.7 *yflM*-encoded protein expression in different combinations of vectors and *E.coli* strains



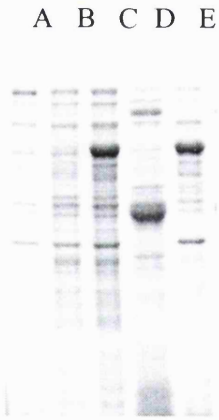
A. Marker; B. Denatured purified *yflM*-encoded protein (YFLM);
B. YFLM in pET21b (supernatant); D. YFLM in pET 21b (pellet);
E. YFLM in pProxHT (supernatant); F.YFLM in pProX HT (pellet);
G. YFLM in pTrc99a (supernatant); H. YFLM in pTrc99a (pellet);
I. YFLM in pCWori (supernatant); J. YFLM in pCWori (pellet).

Figure 3.8 Construction of pCWGST(right) and *yflM* in pCWGST(left)



A. DNA marker; B. pCWGST; C to H. clones of *yflM* in pCWGST;
F. negative; I to K. GST fragment in pCWori.

Figure 3.9 *yflM*-GST fusion protein expressed in *E.coli*



A. Uninduced expression; B and D. supernatant from two colonies;
C and D. Pellet fraction from the two colonies.

3.6. *yflM* expression in *B. subtilis* (Northern and Western blots)

To determine whether *yflM* is expressed in *B. subtilis*, Northern blotting experiments were carried out. Growth of bacterial cultures was as described in section in 3.4. Extraction and purification of RNA, Northern blotting and hybridisation were as described in section 2.8.1, 2.8.2, and 2.8.3.

Figure 3.10 shows the Northern blot result. *yflM* expressed in *E. coli* in plasmid pET28a was used as a positive control. It is clear from the blot that the size of the *yflM* mRNA band in the *E. coli* control track appears slightly larger than the native *yflM* mRNA band in *B. subtilis*. Reasons for this slight discrepancy may be (i) the additional sequence at the start of transcription in the pET28a vector and (ii) there is an additional 60bp (encoding an extra 20 amino acid coding sequence) before *yflM* in pET28a.

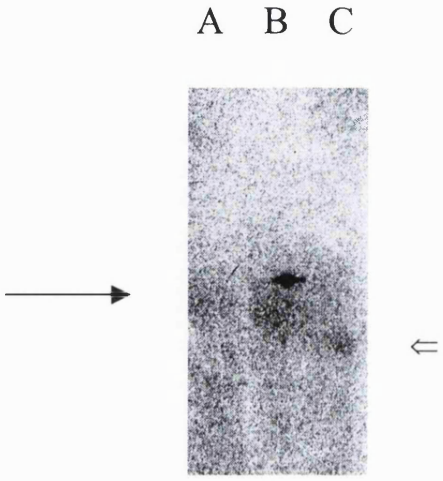
For protein expression studies, two peptides encoded by *yflM* were used to immunise rabbits for raising antiserum. The peptide sequences were:

1) SCSLTAACEELGWRGERTDF (*yflM*121) and
2) HTAASQFKRFEEQEEEAGRK (*yflM*276).

Western blot experiments were carried out with these antibodies to test *yflM* expression in *E. coli* and *B. subtilis*. The *E. coli* expression is discussed in section 2.9.5, and *B. subtilis* culture is described in section 3.4. To determine protein solubility, the supernatant and pellet cell fractions were separated by 100,000 xg centrifugation at 4°C for 1 hour following disruption of *E. coli* and *B. subtilis* cultures by sonication.

Figure 3.11 shows the Western blot results using the two antibodies. When antibody *yflM*121 was used to identify *yflM* expression in *E. coli* and *B. subtilis*, apart from a band (full-length protein) at 40kDa, some additional cross-reacting bands can be identified. There is no band corresponding to the full-length protein detectable in the

Figure 3.10 ***yflM*** **expression in** ***E.coli*** **and in** ***B.subtilis*****: Northern blot**



A. *yflM* in *E.coli*, Rneasy kit extraction;

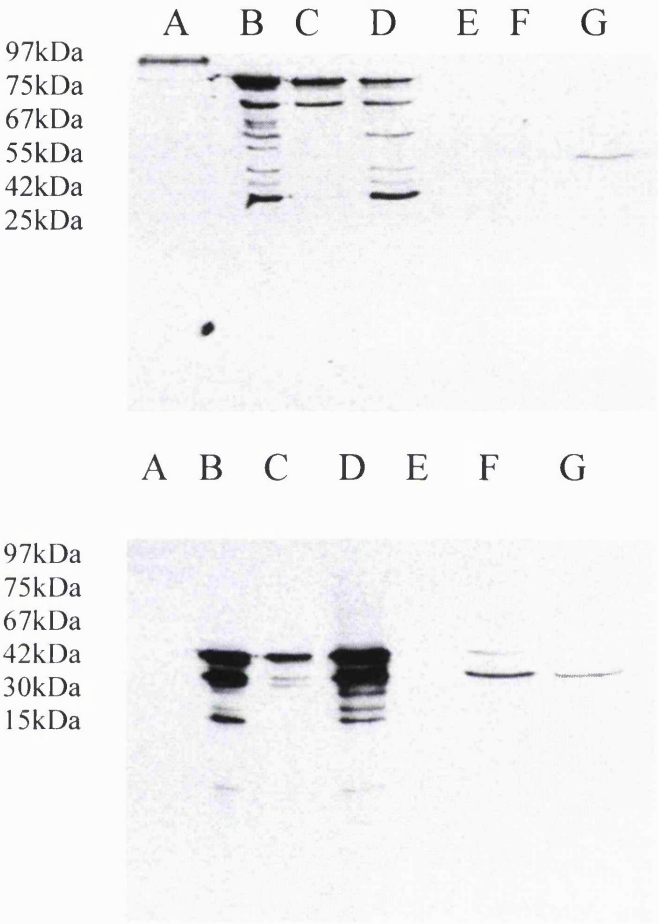
B. *yflM* in *E.coli*, phenol extraction;

C. *yflM* in *B.subtilis* phenol extraction.

→ *yflM* expressed in *E.coli.*

⇐ *yflM* expressed in *B.subtilis.*

Figure 3.11 *yflM* expressed in *E.coli* and *B.subtilis*, top: antiserum *yflM*121; bottom: antiserum *yflM*276



A. Protein marker; B. *yflM* in *E.coli*, whole cell; C. *yflM* in *E.coli*, Supernatant; D. *yflM* in *E.coli*, pellet; E. *yflM* in *B.subtilis*, whole cell; F. *yflM* in *B.subtilis*, supernatant; G. *yflM* in *B.subtilis*, pellet.

soluble fraction of the *E. coli* expressed protein but a full-length protein can be detected in the pellet fraction of *B. subtilis* lysates.

When antiserum *yflM*276 was used, two main bands could be identified, one around 40kDa (corresponding to the full-length protein), the other is between 25 to 30 kDa in both whole cell and insoluble components of the *E. coli* lysate. Interestingly, a band corresponding to the full-length 40kDa protein can be detected by the antibody in the soluble supernatant fraction of *E. coli* expressed protein. This suggests that at least a small fraction of the recombinant *yflM* protein expressed in *E. coli* may be soluble. For the *B. subtilis* protein profile, in both supernatant and pellet components, the main band recognised by the antiserum is between 25 to 30 kDa. In the soluble supernatant fraction, there is a band corresponding to the full-length *yflM*-encoded protein (40 kDa), which can be detected.

3.7. Summary

The function of the *B. subtilis* NOS-like protein encoded by *yflM* is still a mystery. As discussed in the *Bacillus* genome sequencing paper (Kunst, F., *et al.*, 1997) genes with the '*y*' prefix emphasises that the function has not been ascertained.

In this study, the *yflM* gene was cloned by PCR, and its DNA sequence determined. Many attempts were made to generate a soluble expression product, however all of these were unsuccessful. SDS-PAGE and Coomassie blue staining, showed that most of the protein was retained in the insoluble fraction (data was not shown).

Interestingly, using an anti-*yflM* antibody in Western blotting experiments demonstrated that a small fraction of the *yflM*-encoded protein expressed in *E. coli* may have been soluble.

Significantly, most of the protein detected in *B. subtilis* extracts appeared to be smaller than expected. This suggested that either the protein was degraded during the extraction process, or it may naturally be cleaved as part of a maturation process.

CHAPTER 4

CLONING, EXPRESSION, AND PURIFICATION OF SANOS, A NOS LIKE PROTEIN FROM *S.aureus*

CONTENTS

4.1. Introduction

NO plays many different biological roles, including functioning as a neurotransmitter, involved in blood vascular relaxation, and possessing both anti-tumour and anti-microbial activity. NO is also involved in cell respiration and energy generation procedures and in cell-based biochemical reactions. The generation and regulation of NO activity has become an important question in many biological processes.

NO in a cell is produced by a family of nitric oxide synthase enzymes (NOS) through enzymatic conversion of the amino acid L-arginine. NOS is a dual-domain enzyme, and recent research has identified two domains, an oxygenase domain and a reductase domain, that are conjugated together. A number of prosthetic groups are associated with the peptide chain, and include heme, BH_4, L-arg, CaM, FMN, FAD and NADPH. These elements are required for full synthetic activity.

NOS has been identified and characterised in different species, but the existence of NOS in bacteria has not been recognised at molecular level. The aim of the study was to find any possible form(s) of NOS in bacteria using modern molecular biological techniques.

S.aureus is a pathogenic bacterium, which is responsible for infections in different populations, especially in hospital patients. One of the most serious issues of *S.aureus* is its resistance to many common antibiotics. The understanding of the survival, growth and pathogenicity of the bacteria has become an important object of research in modern bacteriology (Novick, R.P., *et al.*, 1993; Wu, S., *et al.*, 1996; Stranden, A.M., *et al.*, 1997; Bellido, J.L.M., *et al.*, 1997; Ling, B., *et al.*, 1998; Perl, T.M., *et al.*, 1998; Archer, G.L., *et al.*, 1998; Noble, W.C., *et al.*, 1998; Sulavik, M.C., *et al.*, 1998; Miyazaki, E., *et al.*, 1998; Foster, T.J., *et al.*, 1998; Clements, M.O., *et al.*, 1998; Su, C., *et al.*, 1998).

The finding of a NOS-like protein in *S.aureus* in this study (this chapter) and its characterisation (next chapter) may be helpful in understanding the role of NO in

Gram-positive bacteria and how this may contribute to pathogenicity.

4.2. SANOS, A bacterial protein with homology to the N-terminus of NOS

NOS enzymes have been already found in many eukaryotic species using molecular biological techniques. Previously, a few papers have discussed the finding of NOS activity in the bacteria *S.aureus* and *Nocardia sp.* These papers have concentrated on protein purification, and limited NOS biochemical assays. To date, molecular evidence for the finding of NOS-like genes in these species is not complete. In fact the only evidence for the finding of any bacterial NOS-like sequence has come from the *B. subtilis* genome-sequencing project (Kunst, F., *et al.*, 1997). Many bacterial genomes have been sequenced, or are in the process of being sequenced. A good place to start the search for NOS-like sequences is from the finished and unfinished microbial genomic projects. Could bacteria have genes encoding NOS or NOS-like proteins?

For the purpose of the work, BLAST was used to search the TIGR database of 'Finished and Unfinished Microbial Genomes'. The search was carried out using human iNOS (as a full-length, protein, and as separate N- and C-terminal domains) as template. During the search, bacterial sequences with considerable identity to the NOS heme domain, and the NOS reductase domain were identified. In fact proteins with identity to both these domains were found in the *S.aureus* genome. **Appendix 2** shows a typical BLAST result after a search for proteins with identity to the NOS heme domain. The protein with a high identity score with the N-terminal heme domain of NOS was given the name SANOS. The protein comprises 358 amino acid residues. When it was aligned with human iNOS and other known NOS sequences (**Figure 4.1**,), the similarity of SANOS and human iNOS was around 60%. The heme binding site sequence was highly conserved between the NOS family enzymes and SANOS (**Figure 4.2**).

Figure 4.1 Alignment of SANOS and human iNOS

```
SANOS ------------------------------------------------------------
HiNOS MACPWKFLFRVKSYQGDLKEEKDINNNVEKTPGAIPSPTTQDDPKSHKHQNGFPQFLTGT 60


SANOS ------------------------------------------------------------
HiNOS AQNVPESLDKLHVTPSTRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIMNSK 120


SANOS --------------MLFKEAQAFIENMY---KECHYETQIINKRLHDIELEIKETGTYTH 43
HiNOS SLTRGPRDKPTPVEELLPQAIEFINQYYGSFKEAKIEEHLA--RLEAVTKEIETTGTYQL 178
                     *: :*  **:: *   **.: * ::   **. :  **: ****


SANOS TEEELIYGAKMAWRNSNRCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLK 103
HiNOS TLDELIFATKMAWRNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIR 238
      * :***:.:******: *****: *..*:*:***. :  :.::. *  **  ***.*:::


SANOS PYITIYAPKDGP----KIFNNQLIRYAGYDN-----CGDPAEKEVTRLANHLGWKGKGTN 154
HiNOS SAITVFPQRNDGKHDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGR 298
      . **::. ::.     :*:*.********:       ****  *.*:*. .**** :  .


SANOS FDVLPLIYQLPNESVKFYEYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGG 214
HiNOS FDVLPLVLQAHGQDPEVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGG 358
      ******: *  .:. :.:* *..*: **.:** :*  :::*.*****:* ::** *::**


SANOS IVYPTAPFNGWYMVTEIGVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYA 274
HiNOS LEFPACPFNGWYMGTEIGVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAA 418
      : :*:.******* ******:: *  ***:**:*.  : :.* .  *: ****:.*:* *


SANOS VYHSFKKEGVSIVDHLTAAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYD 334
HiNOS VLHSFQKQNVTIMDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEML 478
      * ***:*:.*:*:** **::.*  . :** :     ...* **.**:* ::*. :*:


SANOS NTVKDPNFFYKKKESNANFHH---------------------------------------- 347
HiNOS NYVLSPFYYYQIEPWKTHIWQDEKLRPRRREIRFTVLVKAVFFASVLMRKVMASRVRATV 538
     * * .* ::*: :
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Figure 4.2 Alignment of heme binding sites between SANOS and NOSs

```
Murine iNOS          WRNAPRCIGRIQW
Rat nNOS             WRNAPRCIGRIQW
Guinea pig iNOS      WRNAPRCIGRIQW
Human iNOS           WRNAPRCIGRIQW
Dog iNOS             WRNAPRCIGRIQW
Chicken iNOS         WRNAPRCIGRIQW
Mouse nNOS           WRNASRCVGRIQW
Rabbit iNOS          WRNASRCVGRIQW
Bullfrog nNOS        WRNASRCVGRIQW
Human eNOS           WRNAPRCVGRIQW
Guinea pig eNOS      WRNAPRCVGRIQW
Dog nNOS             WRNAPRCVGRIQW
Pig iNOS             WRNAPRCVGRIQW
Bovine eNOS          WRNAPRCVGRIQW
Snail NOS            WRNAPGCIGRSQW
Drosophlia NOS       WRNSSRCIGRIQW
Mosquito NOS         WRNAPRCIGRIQW
Hornworm NOS         WRNATRCIGRIQW
SANOS (S.aureus)     WRNSNRCIGRLFW
                     ***: **:** .*
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Interestingly, a comparison of SANOS with human iNOS (domain structure) demonstrates that the bacterial sequence starts after the putative BH_4 binding site in the human iNOS sequence, and ends before the CaM binding site. (**Figure 4.3**, Titheradge, M.A. *et al.*, 1998). According to this alignment, SANOS may possess the heme and L-arginine binding ability of NOS, but is unlikely to possess BH_4 binding.

In addition to SANOS in *S.aureus*, some N-terminal domain NOS-like proteins were also identified in other bacteria, such as *Bacillus subtilis* (YFLM), *Bacillus anthracis* (Anthra), *Bacillus halodurans* (Bacha) and *Deinococcus radiodurans* (Radiod). In chapter 3, a NOS heme domain-like protein from *B. subtilis*, (encoded by *yflM)* has been described. From BLAST results, it seems that there may be a family of NOS-like proteins in bacteria, and from the data so far, this appears to be confined to Gram-positive bacteria. An alignment of all these bacterial NOS-like sequences with human iNOS (**Figure 4.4**) demonstrates that there is a great deal of apparent heterogeneity in the putative start sites for bacterial NOS-like proteins.

4.3. Amplification of SANOS by PCR, verification by DNA sequencing

4.3.1. PCR amplification of *sanos* from *S.aureus* genomic DNA

A pair of primers was synthesised (Genosys) for PCR experiments to amplify the *sanos* gene. The sequences of the primers are:

5' end primer (i) 5'-CGCATATGGGAGGACACCACCACCACCACCAC
TTATTTAAAGAGGCTCAAGCTTTCATAGAAAACATG-3'

3' end primer (ii) 5'-CGTCTAGATTAATGATGGAAAGGGCACTGG-3'.

Figure 4.3 Domain structure of NOS isoenzymes and SANOS

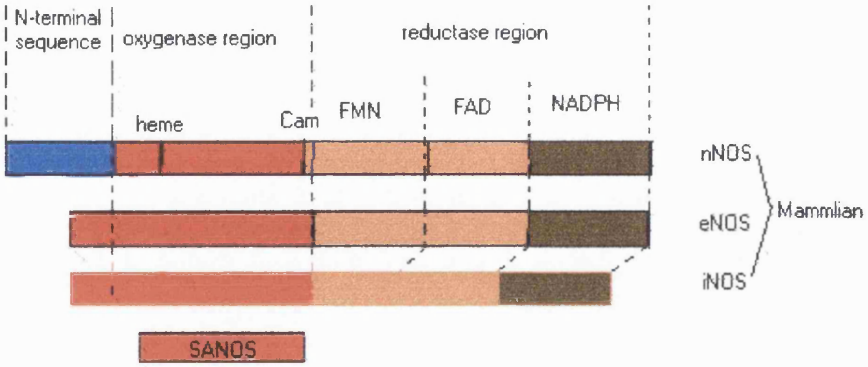


Figure 4.4 Alignment of human iNOS with bacterial NOS-like proteins

```
HiNOS  MACPWKFLFRVKSYQGDLKEEKDINNNVEKTPGAIPSPTTQDDPKSHKHQNGFPQFLTGT 60
Anthra ------------------------------------------------------------
Bacha  ------------------------------------------------------------
Radio  ------------------------------------------------------------
SANOS  ------------------------------------------------------------
YFLM   ------------------------------------------------------------

HiNOS  AQNVPESLDKLHVTPSTRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIMNSK 120
Anthra ------------------------------------------------------------
Bacha  ------------------------------------------------------------
Radio  ------------------------------------------------------------
SANOS  ------------------------------------------------------------
YFLM   ------------------------------------------------------------

HiNOS  SLTRGPRDKPTPVEELLPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTL 180
Anthra ----------------------------------------MKEIQAEIEKTGTYEHTF 18
Bacha  ---------MEEKERLQLEAESFLTKCY-EELG-STGELSKRLEEVRKEIDKTGTYVHTT 49
Radio  --------------MSCPAAAVLTPDMRAFLRRFHEEMGEPGLPARLRAVEEAGLWWPTS 46
SANOS  --------------MLFKEAQAFIENMY-KECHYETQIINKRLHDIELEIKETGTYTHTE 45
YFLM   -------------------------------------VKDRLADIKSEIDLTGSYVHTK 22
                                                :      :. :* :  *

HiNOS  DELIFATKMAWRNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSA 240
Anthra EELVHGSRMAWRNSNRCIGRLFWSKMHILDAREVNDEEGVYHALIHHIKYATNDGKVKPT 78
Bacha  KELAHGARMAWRNSNRCIGRLFWESLHVLDCRHLQTEEEMAEALVDHITYATNDGKILPT 109
Radio  AELTWGAKVAWRNSTRCVGRLYWEALSVRDLRELNTAQAVYEALLQHLDDAFCGGHIRPV 106
SANOS  EELIYGAKMAWRNSNRCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPY 105
YFLM   EELEHGAKMAWRNSNRCIGRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPT 82
        **  .:::****: **:**: *. : : * *       .   :  *:  *   *:: .

HiNOS  ITVFPQRNDGKHDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFD 300
Anthra ITIFKQYQGEENNIRIYNHQLIRYAGYKTEM-GVTGDSHSTAFTDFCQELGWQGEGTNFD 137
Bacha  ISVFRPRHPNKGDVRIWNQQLIRYAGYEEGD-QVIGDPISTKFTQACERLGWSGERTPFD 168
Radio  ISVFGPG------VRLHNPQLIRYA----------DDPINADFVDKLRRFGWQPRGERFE 150
SANOS  ITIYAPKDGPK----IFNNQLIRYAGYDN-----CGDPAEKEVTRLANHLGWKGKGTNFD 156
YFLM   ITIFPPEEKGEKQVEIWNHQLIRYAGYESDG-ERIGDPASCSLTAACEELGWRGERTDFD 141
       *:::           : * ******          .*.    ..     :**  .   *:

HiNOS  VLPLVLQAHGQDP-EVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGL 359
Anthra VLPLVFSIDGKAP-IYKEIPKEEVKEVPIEHPEYP-ISSLGAKWYGVPMISDMRLEIGGI 195
Bacha  VLPLVIQD-GSKPPKWFAVPNESVKEVPLRHPEYEWFAGFQLKWYAVPIVSNMRLEIGGI 227
Radio  VLPLLIEVNGRAE--LFSLPPQAVQEVAITHPVCLGIGELGLRWHALPVISDMHLDIGGL 208
SANOS  VLPLIYQL-PNESVKFYEYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGGI 215
YFLM   LLPLIFRMKGDEQPVWYELPRSLVIEVPITHPDIEAFSDLELKWYGVPIISDMKLEVGGI 201
       :***:              * . : **.: *     :  :  :*:.:* :::* *.:**:

HiNOS  EFPACPFNGWYMGTEIGVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAAV 419
Anthra SYTAAPFNGWYMGTEIGARNLADHDRYNLLPAVAEMMDLDTSRNGTLWKDKALIELNVAV 255
Bacha  HYPAAPFNGWYMGTEIGARNLADEDRYNILPKMAEYMGLSTGKDSTLWKDKALVELNVAI 287
Radio  HLPCA-FSGWYVQTEIAARDLADVGRYDQLPAVARALGLDTSRERTLWRDRALVELNVAV 267
SANOS  VYPTAPFNGWYMVTEIGVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAV 275
YFLM   HYNAAPFNGWYMGTEIGARNLADEKRYDKLKKVASVIGIAADYNTDLWKDQALVELNKAV 261
           . *.***: ***..*:: *  **: *  :.  : : :     : :*:*: *:* *:
```

```
HiNOS   LHSFQKQNVTIMDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLN 479
Anthra LHSFKKQGVSIVDHHTAAQQFQQFEKQEAACGRVVTGNWVWLIPPLSPATTHIYHKPYPN 315
Bacha   LYSYKQEGVSIVDHHTAAKQFARFEQAEQAANRKVTGRWSWLIPPVSPATTHIFHHEYED 347
Radio   LHSFDAAGVKLADHHTVTAHHVRFEEREARAGREVRGKWSWLVPPLSPATTPLWSRRYRA 327
SANOS   YHSFKKEGVSIVDHLTAAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDN 335
YFLM    LHSYKKQGVSIVDHHTAASQFKRFEEQEEEAGRKLTGDWTWLIPPISPAATHIFHRSYDN 321
         :*:.  .*.: ** *.:  .  . . *       . * ** **:* : *  : :

HiNOS   YVLSPFYYYQIEPWKTHIWQDEKLRPRRREIRFTVLVKAVFFASVLMRKVMASRVRATVL 539
Anthra EILKPNFFHK---------------------------------------------------- 325
Bacha   ETVLPNYFYQPAPYESDTF------------------------------------------- 366
Radiod REESPRFVRARCPFHTPTVHASTGHAPTG------------------------------- 356
SANOS   TVKDPNFFYKKKESNANQCPFHH------------------------------------- 358
YFLM    SIVKPNYFYQDKPYE------------------------------------------------ 336
            * :
```

HiNOS:	Human inducible nitric oxide synthase,
Anthra:	NOS heme domain-like protein from *Bacillus anthracis*
Bacha:	NOS heme domain-like protein from *Bacillus halodurans*
Radio:	NOS heme domain-like protein from *Deinococcus radiodurans*
SANOS:	NOS heme domain-like protein from *Staphylococcus aureus*
YFLM:	NOS heme domain-like protein from *Bacillus subtilis*

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

A '6 histidine' coding sequence motif was designed into the N-terminal primer with an *NdeI* site and two extra amino acids, to facilitate purification of the recombinant protein by metal affinity chromatography. The C-terminal primer incorporated an *XbaI* site to facilitate cloning. The *Pfu* DNA polymerase was used to perform PCR. The *S.aureus* genomic DNA was a gift from Dr. Michael Lockyer (Arrow Therapeutics, Carshalton, Surrey). The concentration of the DNA was 0.1 μg/μl. The PCR was carried out according to the condition described in **2.7.12**.

Figure 4.5 shows the PCR product of *sanos*, which was of the expected size (1126 bp). The *sanos* PCR product was cloned into the TOPO vector (**2.7.12**) and its DNA sequence was verified on an Applied BioSystems Sequencer (GlaxoWellcome Medicines Research Centre, Stevenage).

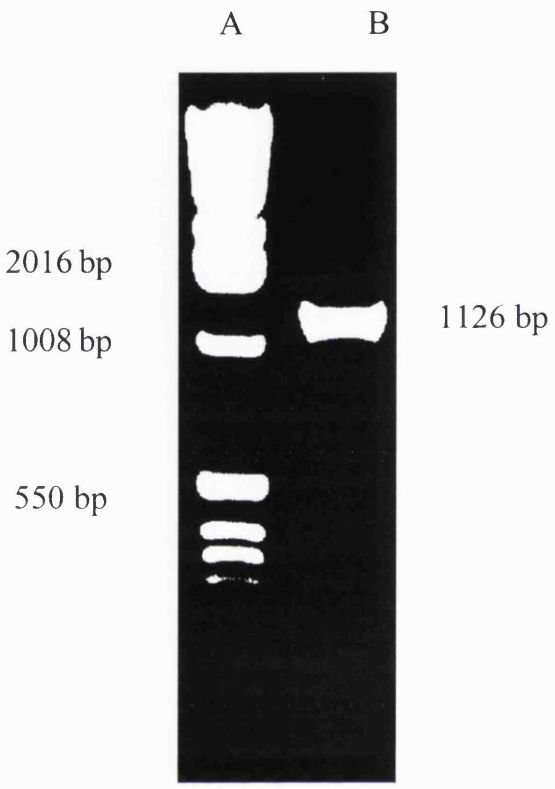
4.3.2. Sequencing verification

Figure 4.6 shows *sanos* DNA and its encoded peptide sequences, which were verified. This result suggests that not only was the sequence of *sanos* correct, but also that the 6 histidine residues (along with two extra amino acids) had been incorporated successfully in the N-terminal of the *sanos* PCR product.

4.4. SANOS expression in *S.aureus*

Northern blotting was carried out for the detection of expression of *sanos* in *S.aureus*. The *S.aureus* used was purchased from the Public Health Laboratory Service (PHLS) in London. It was grown in Brain Heart Infusion broth or Tryptic Soy Broth. *S.aureus* RNA was extracted according to the procedure described in **2.8.1**. The de-natured agarose gel electrophoresis, and Northern blot and hybridisation procedures were described in **2.8.2**, and **2.8.3**.

Figure 4.5 PCR product of SANOS



A. DNA marker; B. SANOS.

Figure 4.6 SANOS DNA and peptide sequences

```
ATG TTA TTT AAA GAG GCT CAA GCT TTC ATA GAA AAC ATG TAT AAA
 M   L   F   K   E   A   Q   A   F   I   E   N   M   Y   K    15

GAG TGT CAT TAT GAA ACG CAA ATT ATC AAT AAA CGT TTA CAT GAC
 E   C   H   Y   E   T   Q   I   I   N   K   R   L   H   D    30

ATT GAA CTA GAA ATA AAA GAA ACT GGG ACA TAT ACA CAT ACA GAA
 I   E   L   E   I   K   E   T   G   T   Y   T   H   T   E    45

GAA GAA CTT ATT TAT GGT GCT AAA ATG GCT TGG CGT AAT TCA AAT
 E   E   L   I   Y   G   A   K   M   A   W   R   N   S   N    60

CGT TGC ATT GGT CGT TTA TTT TGG GAT TCG TTA AAT GTC ATT GAT
 R   C   I   G   R   L   F   W   D   S   L   N   V   I   D    75

GCA AGA GAT GTT ACT GAC GAA GCA TCG TTC TTA TCA TCA ATT ACT
 A   R   D   V   T   D   E   A   S   F   L   S   S   I   T    90

TAT CAT ATT ACA CAG GCT ACA AAT GAA GGT AAA TTA AAG CCG TAT
 Y   H   I   T   Q   A   T   N   E   G   K   L   K   P   Y   105

ATT ACT ATA TAT GCT CCA AAG GAT GGA CCT AAA ATT TTC AAC AAT
 I   T   I   Y   A   P   K   D   G   P   K   I   F   N   N   120

CAA TTA ATT CGC TAT GCT GGC TAT GAC AAT TGT GGT GAT CCT GCT
 Q   L   I   R   Y   A   G   Y   D   N   C   G   D   P   A   135

GAA AAA GAA GTT ACA CGC TTA GCA AAT CAC TTA GGT TGG AAA GGA
 E   K   E   V   T   R   L   A   N   H   L   G   W   K   G   150

AAA GGT ACT AAT TTT GAC GTG TTA CCA CTG ATT TAC CAA TTA CCT
 K   G   T   N   F   D   V   L   P   L   I   Y   Q   L   P   165

AAT GAG TCA GTT AAA TTT TAC GAA TAT CCT ACT TCA TTA ATT AAA
 N   E   S   V   K   F   Y   E   Y   P   T   S   L   I   K   180

GAA GTA CCT ATT GAA CAT AAT CAT TAT CCA AAA TTA AGA AAA TTG
 E   V   P   I   E   H   N   H   Y   P   K   L   R   K   L   195

AAC TTA AAA TGG TAT GCA GTC CCT ATC ATT TCC AAT ATG GAC TTA
 N   L   K   W   Y   A   V   P   I   I   S   N   M   D   L   210

AAA ATC GGT GGC ATT GTA TAT CCA ACT GCA CCC TTT AAC GGT TGG
 K   I   G   G   I   V   Y   P   T   A   P   F   N   G   W   225

TAT ATG GTA ACT GAA ATT GGC GTA CGT AAC TTT ATT GAT GAT TAC
 Y   M   V   T   E   I   G   V   R   N   F   I   D   D   Y   240

CGT TAC AAT TTA CTA GAA AAA GTT GCA GAT GCG TTT GAA TTT GAT
 R   Y   N   L   L   E   K   V   A   D   A   F   E   F   D   255

ACA CTT AAA AAT AAT TCA TTT AAT AAA GAT CGA GCA CTT GTT GAA
 T   L   K   N   N   S   F   N   K   D   R   A   L   V   E   270

TTG AAC TAT GCT GTG TAT CAT TCC TTT AAA AAA GAA GGC GTA TCA
 L   N   Y   A   V   Y   H   S   F   K   K   E   G   V   S   285

ATT GTC GAT CAT TTG ACC GCT GCA AAG CAA TTC GAA CTA TTC GAA
 I   V   D   H   L   T   A   A   K   Q   F   E   L   F   E   300

CGT AAC GAA GCA CAA CAA GGT CGT CAA GTT ACC GGA AAA TGG TCT
 R   N   E   A   Q   Q   G   R   Q   V   T   G   K   W   S   315

TGG CTA GCA CCG CCA TTA TCT CCA ACA TTG ACG TCA AAT TAT CAT
 W   L   A   P   P   L   S   P   T   L   T   S   N   Y   H   330

CAC GGA TAT GAC AAT ACA GTA AAA GAT CCA AAC TTT TTC TAT AAA
 H   G   Y   D   N   T   V   K   D   P   N   F   F   Y   K   345

AAG AAA GAA TCA AAT GCT AAC CAG TGC CCT TTC CAT CAT TAA
 K   K   E   S   N   A   N   Q   C   P   F   H   H   *       358
```

Figure 4.7 shows the Northern blot result. The *sanos* PCR product was used as probe (**2.7.14**). *E. coli* expressing SANOS was used as a positive control. Analysis of the result suggests that the *sanos* mRNA expressed in *S.aureus* is approximately the same size as the expressed product in *E. coli.*

4.5. SANOS expression in *E. coli* and purification by FPLC

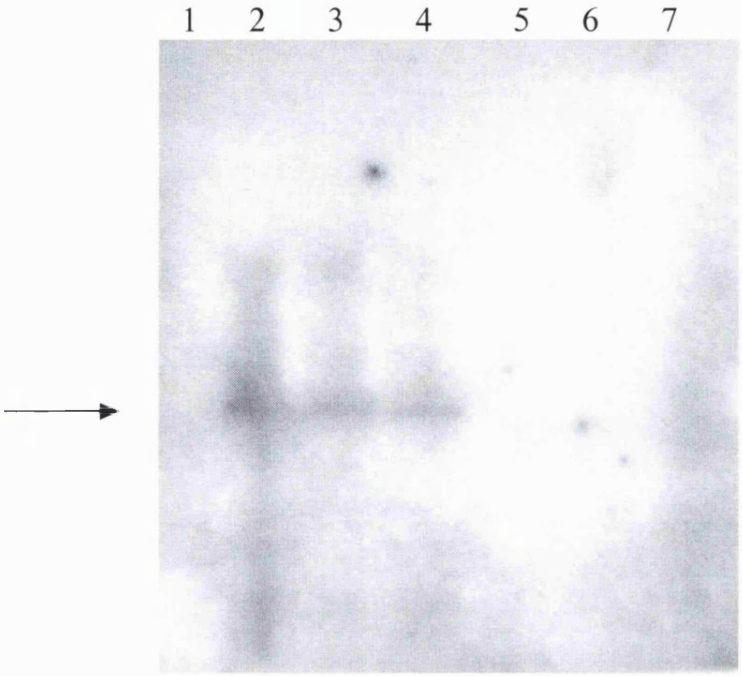
The expression vector pCWori was used to express SANOS in *E. coli.* This vector was selected as it has been used for the expression of other heme containing proteins, e.g., cytochrome P450 and NOS proteins in *E. coli* (Barnes, H.J., *et al.*, 1991; McMillan, K., *et al.*, 1995; Martasek, P., *et al.*, 1996; Rodriguez-Crespo, I., *et al.*, 1996; Licad-Coles, E., *et al.*, 1997; Parikh, A., *et al.*, 1997). SANOS was cloned into *NdeI/XbaI* sites in the vector. The recombinant plasmid was transformed into BL21 (DE3) for expression.

The expression of SANOS in *E. coli* was discussed in section **2.9.5**. After 48 hours induction, the OD_{600} of the culture reached 23. Interestingly, the cell pellet obtained following centrifugation was dark brown in colour, (consistent with the findings of other recombinant heme-domain proteins). The purification by FPLC was as described in section **2.9.7**. The protein eluted from the column was dark brown in colour, consistent with the behaviour of a heme protein.

The eluted SANOS was concentrated by ammonium sulphate precipitation (**2.9.8**) and could be stored at 10 mg/ml at -80°C without precipitation until needed. **Figure 4.8** shows the purified SANOS.

For comparative control experiments, the heme domain of murine iNOS (amino acid 66 to 498) was expressed and purified in the same way as SANOS following the published protocol (Rodriguez-Crespo I *et al.*, 1996). Interestingly the OD_{600} of induced cultures of expressed murine heme domain protein reached a very high density OD600 = 23.

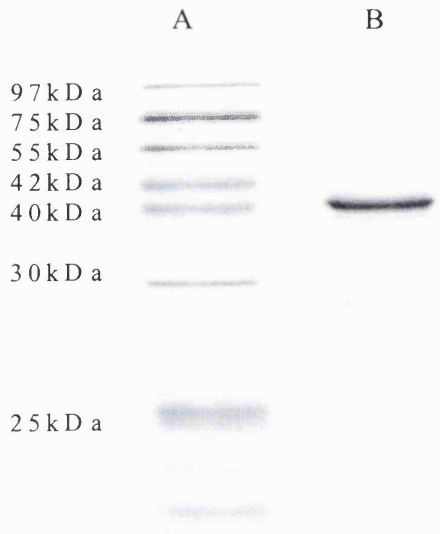
Figure 4.7 The expression of *sanos* in *S.aureus*—Northern blot



1.Marker; 2. SANOS expressed in *E.coli*; 3, 4. SANOS in *S.aureus* (RNA extracted by phenol:chloroform); 5, 6, 7. SANOS in *S.aureus* (RNA extracted by RNAeasy kit).

→ *Sanos* expressed in *E.coli* and *S.aureus*.

Figure 4.8 Purification of SANOS



A. Protein Marker; B. Purified SANOS.

4.6. Spectrum of SANOS

Figure 4.9 shows the spectrum of purified SANOS and murine iNOS heme domain. The two proteins possess similar diagnostic heme peaks at 415 nm, 540 nm and 650 nm.

4.7. Heme group determination

The heme group in SANOS and in the heme domain of murine iNOS, was determined as described in **2.10.1.**

According to Klatt P (1996), the spectrum of the murine iNOS heme domain (**Figure 4.10**) shows a typical ferroprotoporphyrin IX characterisation with three peaks at 420, 524 and 556 nm respectively. Interestingly, SANOS also gives the same three peaks. These findings support the hypothesis that SANOS has similar properties to the murine iNOS heme domain.

4.8. Construction of a SANOS allelic replacement vector

For the further study of SANOS function in *S.aureus*, a recombinant plasmid was constructed for the deletion of the *sanos* gene from *S.aureus* genome.

The pBC SK(-) phagemid was used as a vector. Two pairs of primer were designed according to the 5' and 3' flanking sequences of the *sanos* gene (identified from BLAST). The sequences of the primers are:

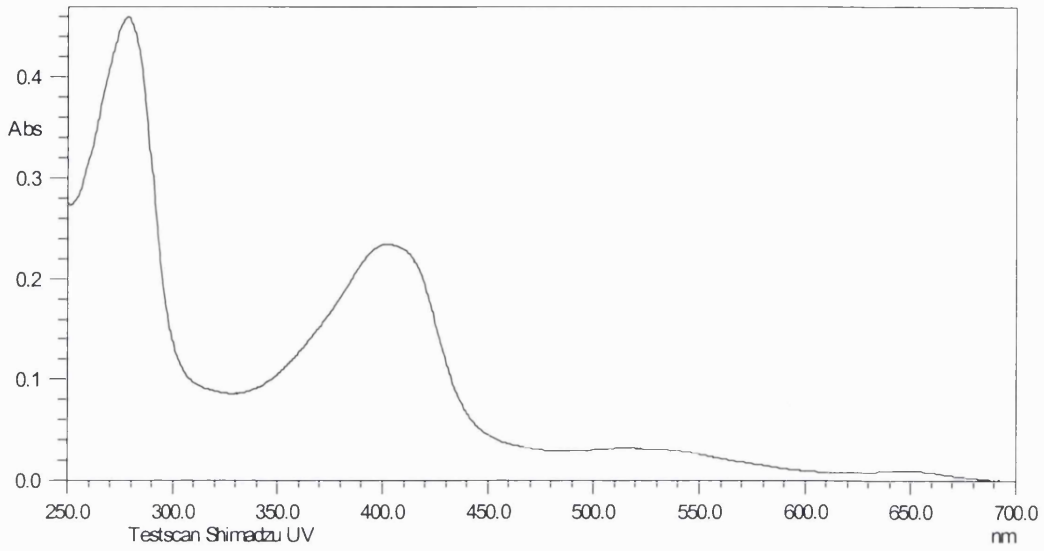
Upstream primers:

5' primer: 5'-TATGGTACCGACGAATTCTGCTAGCCTTTG-3'

3' primer: 5'-CAAGCTTCAACACCTCGCTTTATATTATAG-3'

A *KpnI* site was designed into the N-terminal end of the 5' primer and a *HindIII* site into the 5' end of the 3' primer.

Figure 4.9 Spectrum of SANOS (above) and murine iNOS heme domain (lower).



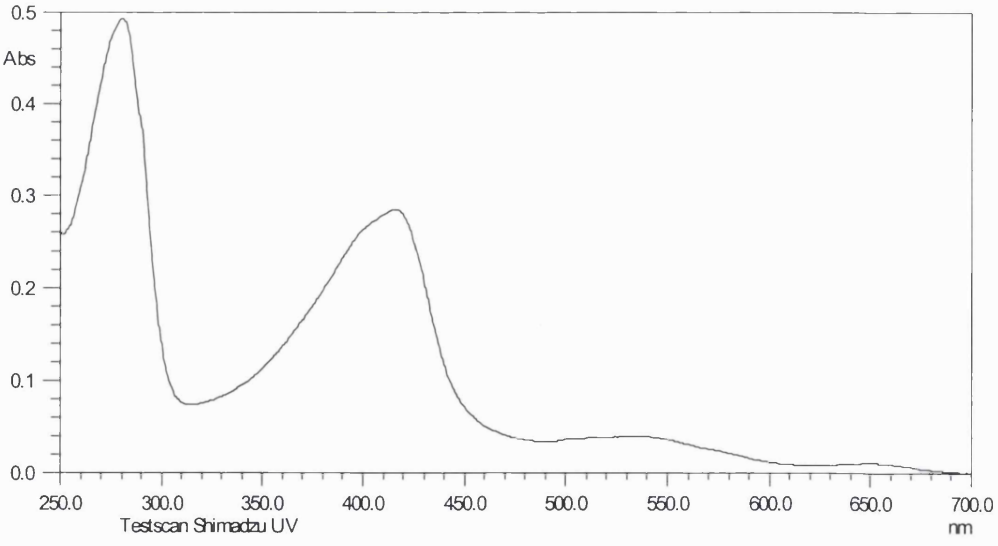
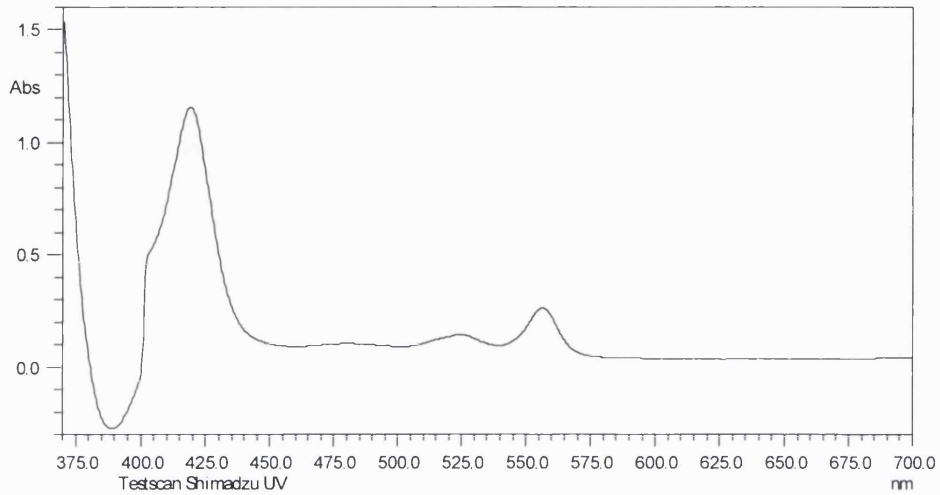
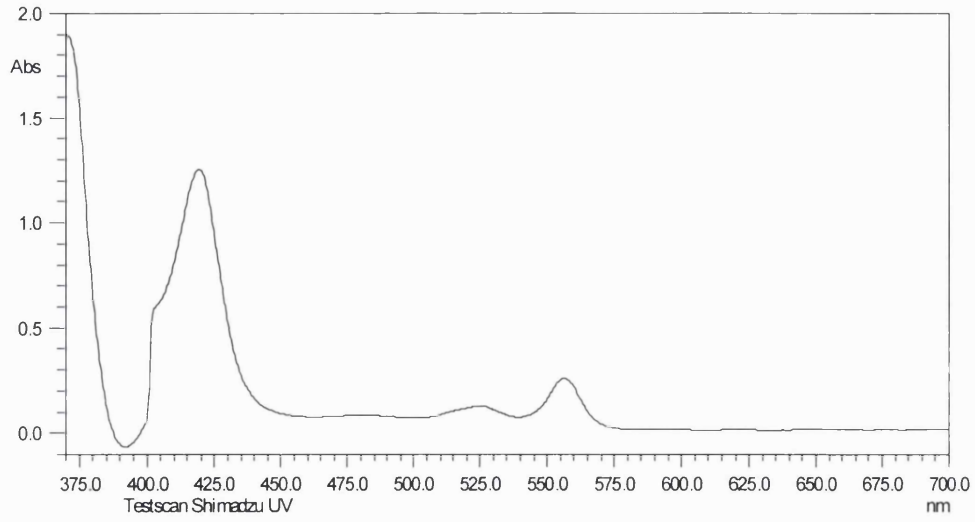


Figure 4.10 Heme group determination of SANOS (above) and murine iNOS heme domain (below)





Downstream primers:

5' primer: 5'-CGAAGCTTGTTAGTAGAGGTGTAGCATATG -3'

3' primer: 5'-ATTCTAGACGCCACTACCTAAAGGTGCAATTG-3'

A *HindIII* site was designed into the 5'-end end of the 5' primer and an *XbaI* site into 5'-end of the 3' primer.

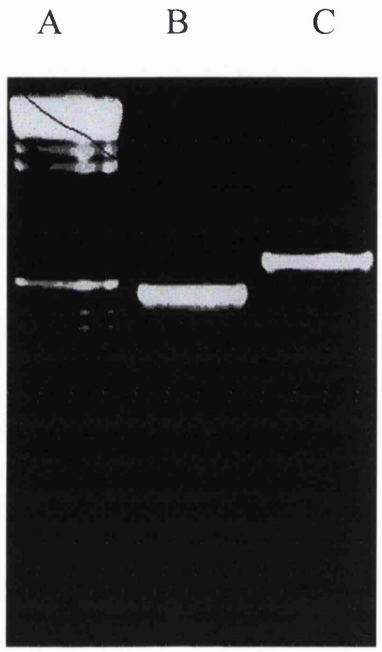
The *S.aureus* genomic DNA was extracted by phenol and precipitated by ethanol (**2.7.4**). The DNA was kept at 0.1mg/ml in water.

Figure 4.11 shows the PCR products of the up - and downstream flanking fragments of the *sanos* gene. These fragments showed the expected sizes. After being recovered from agarose gels, the PCR products were cut by restriction enzymes (*KpnI/HindIII* for the upstream fragment, and *HindIII/XbaI* for the downstream fragment), and cloned into the pBC SK (-) phagemid vector, which was cut by the same enzymes. **Figure 4.12** shows the right-hand fragments from the recombinant plasmid.

A Tetracycline-resistance cassette was cut out by the restriction enzyme *HindIII* from plasmid pT181 (Projan, S.J., *et al.*, 1995; a gift from Dr. Michael Lockyer, Arrow Therapeutics, Carshalton, Surrey). As shown in **Figure 4.13**, there are three *HindIII* sites in the pT181 plasmid. The whole plasmid was cut into three pieces and the tetracycline cassette was in the largest band. The tetracycline cassette was cloned into the recombinant plasmid, (containing the up - and downstream fragments of SANOS) in the pBC SK (-) phagemid vector.

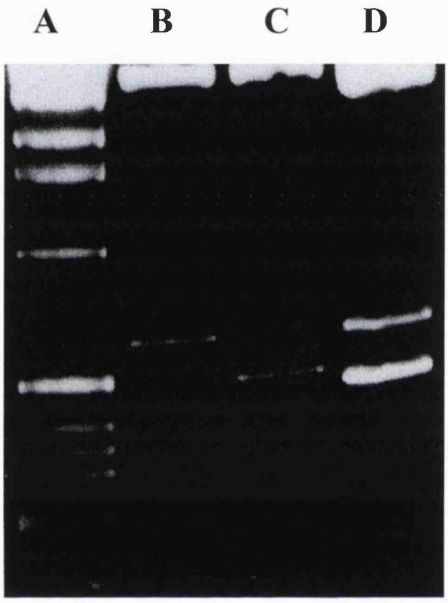
Figure 4.14 shows the screening of the final recombinants. As there are other *KpnI* and *XbaI* sites in the up- and downstream fragments of SANOS, the restriction enzyme digestion showed a multi-band mixture. Two out of the six clones suggested correct insertion.

Figure 4.11 The PCR products of 5' and 3' flanking regions of the *sanos* gene



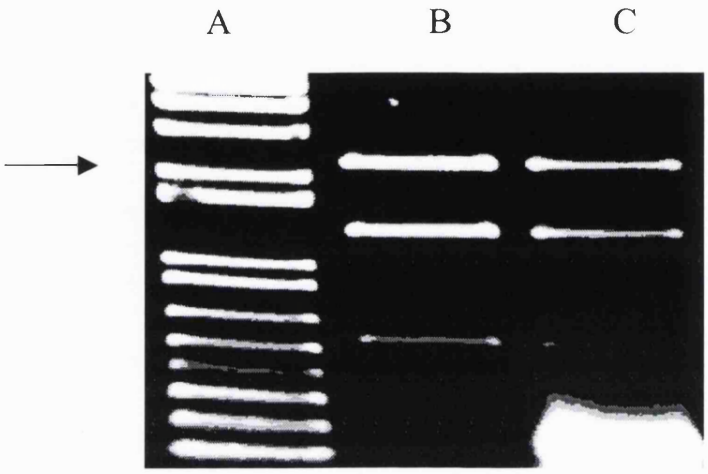
A. DNA lader; B. downstream fragment; C. upstream fragment.

Figure 4.12 Construction 5' and 3'-flanking regions of sanos into pBC SK(-)



A. DNA marker; B. downstream clone; C. upstream clone; D. dual clone.

Figure 4.13 The tetracycline cassette from pT181

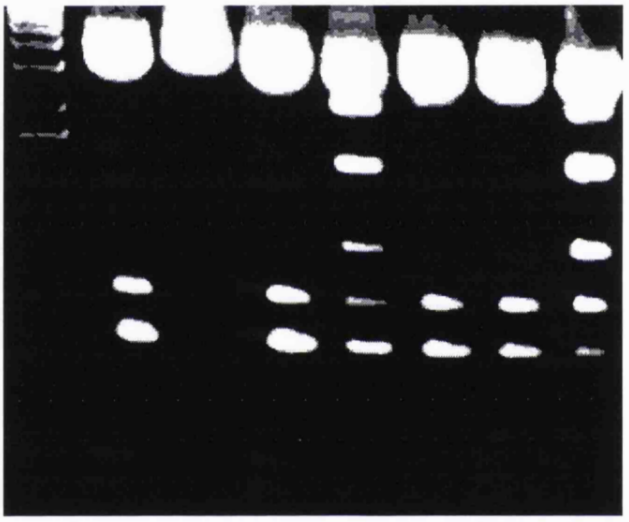


A. DNA 1 kb lader; B,C. pT181 cut by *HindIII*, the arrow indicates the

tetracycline resistance containing fragment.

Figure 4.14 Final allelic replacement plasmid

A B C D E F G H



A. DNA 1 kb ladder. B. sanos 5' and 3' flanking fragments in pBCSK.
C to H. 6 clones of the final constructionwith the tetracycline cassette.

4.9. Summary

A gene encoding a NOS heme-domain like protein was recognised and isolated successfully from *S.aureus*. Because of the similarity with NOS, this protein was called SANOS.

The *sanos* gene encoding SANOS was found in the unfinished genomic sequence of *S.aureus* by BLAST. When the DNA sequence was translated into protein, the length of SANOS was 358 amino acids. The sequence from amino acid residues 62-74 of SANOS, (WRNSNRCIGRLFW) is very conserved with the heme-binding domain (WRNAPRCIGRIQW) found in human iNOS. As the above sequence is considered as the heme-binding site in different NOS enzymes (**Figure 4.3**) the motif alignment data might suggest the first clue that SANOS is a heme-containing protein.

When the SANOS protein sequence was aligned with the three mammalian NOS isoforms (**Appendix 5**), SANOS was shown to align after the BH_4 binding site. Interestingly the SANOS protein alignment ends before the calmodulin-binding site present in mammalian NOS sequences. These results suggest that SANOS may not be able to bind BH_4 or CaM. **Figures 4.2, and 4.3**, demonstrate the possibility that SANOS may be a heme domain-like protein.

One of the features of the study was the primer used to amplify SANOS from the genomic DNA of *S.aureus*. For purification purposes, 6-histidine codons (and two extra 'protection 'amino acids) were designed into the N-terminal primer. From the sequencing results, the PCR product amplified using *Pfu* DNA polymerase shows not only the correct sequence according to the BLAST result, but also shows that the designed histidines and two 'protection' amino acids were incorporated successfully.

When SANOS was expressed in *E. coli*, a significant component of the recombinant protein was soluble. The protein was brown in colour and capable of storing in a reasonable concentration.

Analysis of the spectral properties of the protein suggested that SANOS is a heme protein, very similar to the murine iNOS heme domain.

For further functional studies on SANOS, a recombinant plasmid was constructed to permit allelic replacement of SANOS in the *S.aureus* genome. A 621 base pair fragment 5' to the coding region, and a 465 base pair fragment 3' to the coding region were amplified by PCR and cloned into pBC SK (-) phagemid vector with a tetracycline cassette between the two fragments.

CHAPTER 5

CHARACTERISATION OF SANOS

CONTENTS.

5.1. Introduction

SANOS was expressed in *E. coli* and purified by FPLC. Spectral absorbance analysis suggested that SANOS may be a heme binding protein. As SANOS corresponds to the heme domain of NOS, compound (inhibitor) binding and H_2O_2 oxidation tests were carried out to measure SANOS function. Controls for the experiment were purified murine iNOS heme domain (amino acids 66 to 499) with an N-terminal 6×his tag, and purified rat nNOS heme domain (amino acids 221 to 724, N-terminal EEF tagged). The SANOS stock concentration was maintained at 13 μg/μl, the murine iNOS heme domain at 18.6 μg/μl, and the rat nNOS heme domain at 10 μg/μl.

5.2. Optical difference spectrophotometry

In attempting to determine the role of the heme domain in NOS, McMillan, M., (1993) and colleagues have utilised substrate perturbation difference spectrophotometry for the detection of substrate interaction and determination of binding constants with various cytochrome P450 molecules. The appearance of difference spectra due to heme perturbation by the putative substrates or inhibitors constitutes direct evidence that the heme prosthetic group of rat brain NO synthase binds initially with the substrate, presumably prior to serving as the monooxygenase reaction centre in this enzyme.

Substrate perturbation difference spectrophotometry measurements of NOS were conducted in the absence of calcium/CaM and added BH_4. Optical spectra were recorded using a Shimadzu Model 2401 UV/visible dual-beam spectrophotometer with 1 ml masked quartz cuvettes. Titration experiments were performed at room temperature. All substrate were dissolved in water and the final sample volume changes were<2%. Samples of 1 ml of heme domain were placed in the cuvettes, the absorbance difference was adjusted to zero, and a baseline was recorded. Difference spectra were recorded after each addition at 0.2 nm intervals, from 300 to 500 nm, using a 2 nm slit width. The spectral binding constants, κ_s, were determined from the χ-intercept of a double-reciprocal plot of the difference in the respective peak to

trough absorbance verses the perturbant concentration. Linear transformation of titration data was performed by linear regression analysis.

5.3. Imidazole shift

The main procedure was described in 2.9.2. Briefly, in a 1 ml cuvette, adding in order: 1 ml PBS buffer, 10 μl protein sample, 1 μl 100 mM DTT, 1 μl 100 mM BH_4. After mixing, the spectral analysis was carried out from 300 nm to 500 nm in a Perkin-Elmer Lambda 7 UV/VIS spectrophotometer (GlaxoWellcome) and repeated in an UV-2401 PC, UV-VIS recording spectrophotometer, SHIMADZU (Wolfson Institute). Imidazole was added in a concentration range from 0 μM to 350 μM. Using this methodology, the Ki measurement of imidazole binding to SANOS is 235 μM compared with the Ki for murine iNOS heme domain of 81.5 μM.

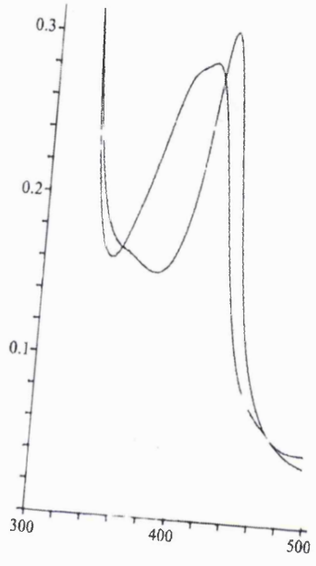
Figure 5.1 shows the results of the imidazole shift assays. The results suggest that both proteins (SANOS and murine iNOS) give similar titration, although the affinity of SANOS for imidazole is lower than murine iNOS heme domain. The Ki of rat nNOS for imidazole (133 μM) is intermediate between the SANOS and iNOS values.

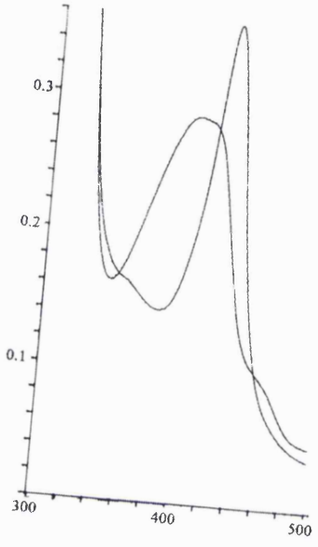
5.4. SANOS binds to L-arginine

Comparing SANOS by sequence alignments with other NOS N-terminal domains suggests that SANOS will be capable of binding heme and L-arginine, but not BH_4. Experimentally, both the heme group determination test, and the imidazole binding assay support this hypothesis.

The ability of SANOS to bind L-arginine was analysed by the procedure described in 4.2. **Figure 5.2** shows the result of L-arg binding to the two proteins (SANOS and the murine iNOS heme domain). The peak at 424 nm raised by imidazole, is shifted to 396 nm for both proteins following L-arg binding. The measurement was carried out in the presence of BH_4. Interestingly, SANOS shows a similar Ki value for arginine as the murine iNOS heme domain, (15 μM as compared with 19 μM). This

Figure 5.1 Imidazole shift of SANOS(left) and murine iNOS(right)

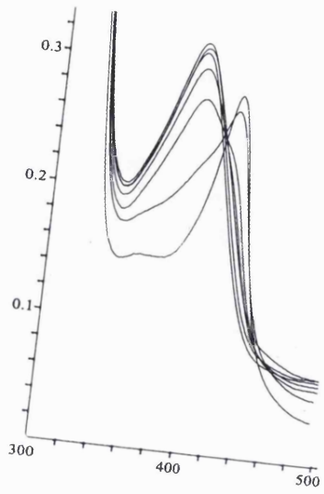


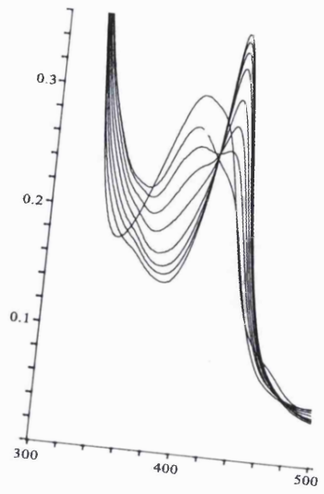


Kd= 235.4 (±38.8) μM

Kd= 81.5 (±15.1) μM

Figure 5.2 L-arginine binding to SANOS (left) and murine iNOS(right)





Kd= 15.7 (±1.3) μM

Kd=19.4 (±1.7) μM

value is much higher than that measured for the nNOS heme domain, (2.9 μM; **Table 5.1**).

For further measurements, the spectral analysis was determined in the absence of imidazole. **Figure 5.3** shows the results of SANOS binding to L-arg with and without BH_4.

According to the spectral analysis, the binding of SANOS to L-arg is independent of exogenous BH_4. This finding supports the protein alignment data that suggested that the BH_4 binding site was missing from the SANOS protein. The peak at 395nm is not affected by BH_4, but the trough of the spectrum is varied from 350nm to 320nm. Murine iNOS does not show similar behaviour.

To support the L-arginine binding data, and to demonstrate specificity, binding assays were set up with D-arginine and L-lysine. Binding assays were carried out as described in section 4.3. **Figure 5.4** shows that SANOS, like the murine iNOS heme domain, binds to L-arginine specifically.

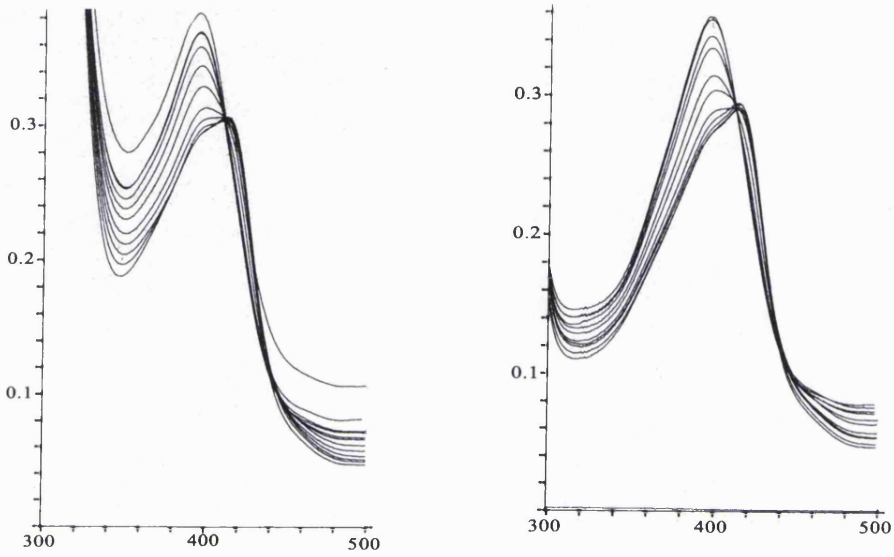
5.5. SANOS binds to the NO synthesis intermediate N^G-hydroxy-L-arginine (NOHA)

NOHA is an NO synthetic intermediate and can be converted to NO and citrulline by NOS isoforms (Stuehr, D.J., *et al.*, 1991; Korth, H., *et al.*, 1994; Campos, K.L., *et al.*, 1995; Ghosh, D.K., *et al.*, 1995; Clague, M.J., *et al.*, 1997; Abu-Soud, H.M., *et al.*, 1997; Moali, C., *et al.*, 1998; Witteveen, C.F.B., *et al.*, 1998). **Figure 5.5** shows the spectrum of NOHA binding to SANOS and iNOS. The two proteins have very similar Ki values with respect to NOHA binding (3.2 μM for SANOS and 4.6 μM for iNOS).

5.6. SANOS binds to NOS inhibitors

The substrate analogue inhibitor, N^G-nitro-L-arginine (NA) has been intensively

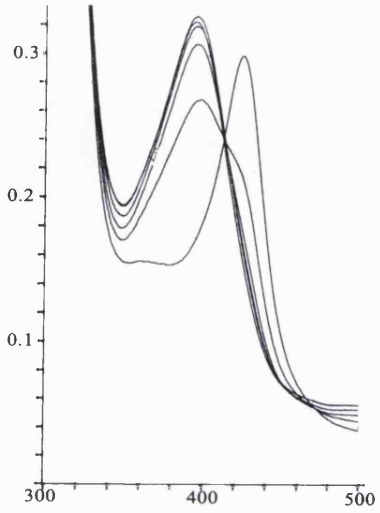
Figure 5.3 L-arginine binds to SANOS: with BH_4 (right), and without BH_4(left)

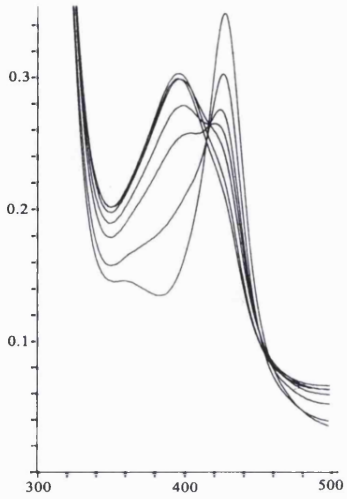


Kd=17.9 (±2.1) μM

Kd=19.6 (±1.5) μM

Figure 5.5 NOHA binding to SANOS(left) and iNOS(right)





Kd=3.2 (±0.2) μM

Kd=4.7 (±0.5) μM

studied (Rondouin, G., *et al.*, 1993, Furfine, E.S., *et al.*, 1993, Klatt, P., *et al.*, 1994, Alderton, W.K., *et al.*, 1998). NA shows a competitive binding pattern with L-arg to NOS and shows greater inhibition of nNOS than iNOS (Furfine, E.S., *et al.*, 1993). In this study, a 60-fold variation between iNOS and nNOS heme domain inhibition was found as compared with the full-length nNOS (Furfine, E.S., *et al.*, 1993). SANOS is less able to bind NA than iNOS. **Figure 5.6** shows NA binding to SANOS and iNOS.

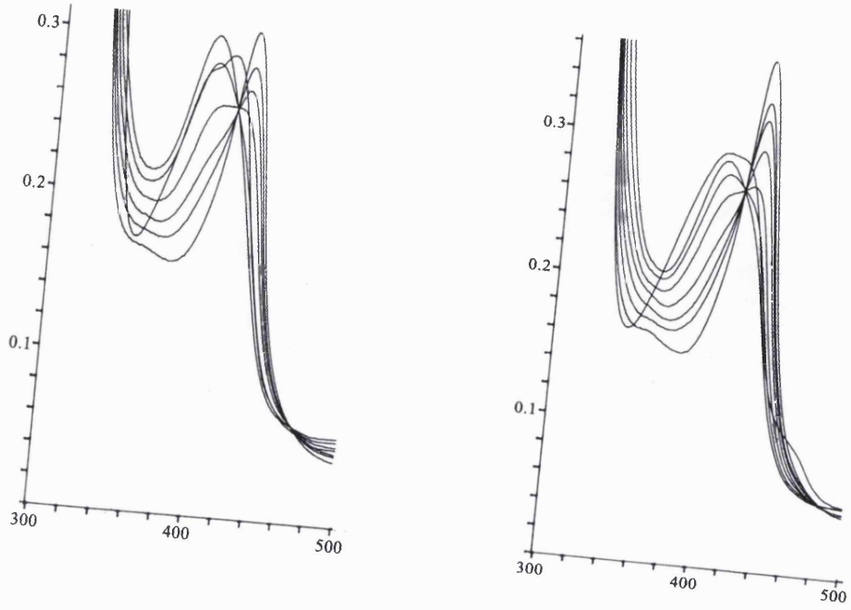
Another arginine analog, N^{ω}-monomethyl-L-arginine (L-NMMA) was studied, like NA, this compound inhibits NOS activity by occupying the L-arg binding site, and the inhibition can be reversed by excess L-arg (Griffith, O.W., *et al.*, 1995). The binding of L-NMMA has been reported to be variable between different NOS isoforms. In this study, L-NMMA shows a different binding pattern comparing iNOS with SANOS, (5.4 μM compared with 9.1 μM; **Table 5.1** and **Figure 5.7**). These values for both iNOS and SANOS are much higher than the binding Ki for nNOS (0.48 μM). In conclusion, SANOS demonstrates a binding to L-NMMA that is more like iNOS rather than the constitutive nNOS.

The binding of the inhibitor 1400W was measured for the three NOS-domains (SANOS, iNOS and nNOS). For SANOS, iNOS and nNOS, the Ki values are 9.1 μM, 6.4 μM, 3.6 μM, respectively. **Figure 5.8** shows the spectrum of 1400W binding comparing SANOS and murine iNOS.

The binding of the three NOS-domains was similar for 1400W; the binding to SEITU and L-NIL is very different for the three proteins. **Figure 5.9** and **5.10** show the results of the binding assays. The Ki for SEITU binding to SANOS (5.7 μM) is much higher than nNOS (0.13 μM), and iNOS (0.39 μM). For L-NIL binding, SANOS has a Ki of 60 μM, and this is also much higher than iNOS (9.4 μM), and nNOS, (3.6 μM).

Table 5.1 summarises the binding results of SANOS, murine iNOS and rat nNOS heme domains.

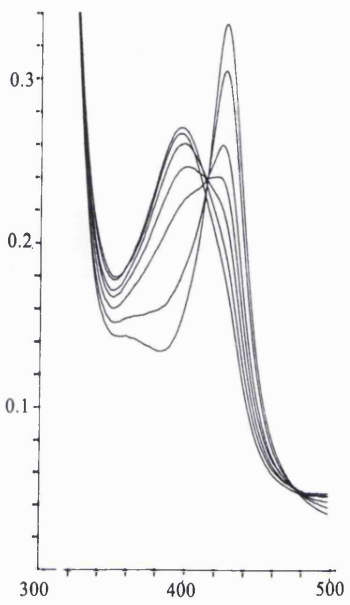
Figure 5.6 NA binding to SANOS (left) and iNOS (right)



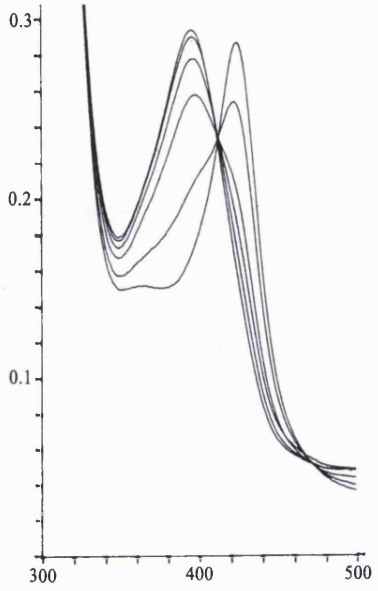
Kd=7.5 (±0.4) μM

Kd=4.9 (±0.5) μM

Figure 5.7 LNMMA binding to SANOS (left) and iNOS (right)

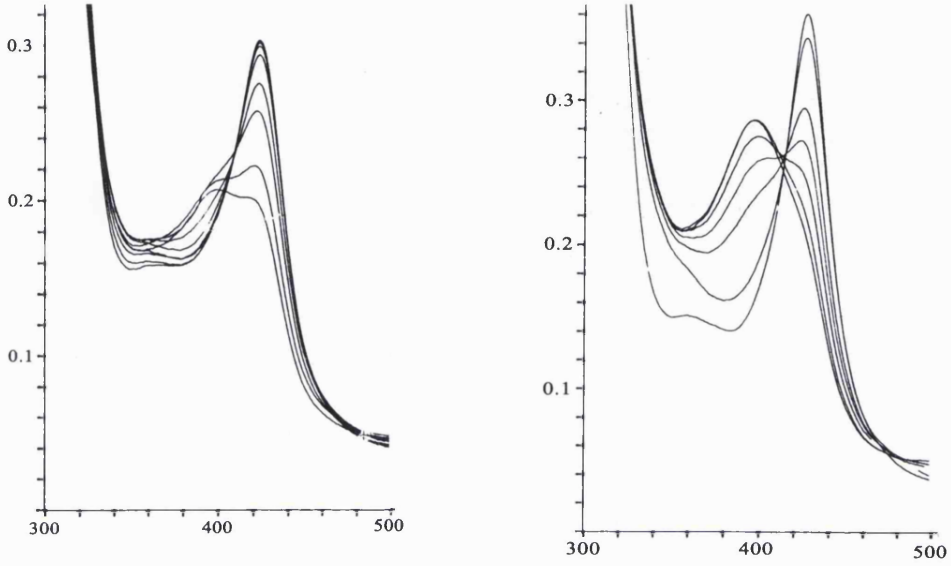


Kd=9.1 (±0.5) μM



Kd=5.4 (±0.4) μM

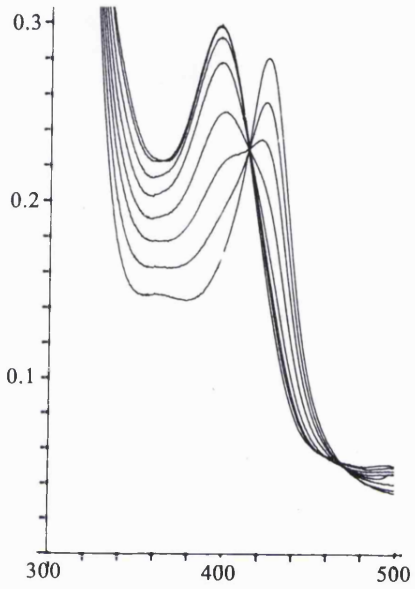
Figure 5.8 1400W binding to SANOS (left) and iNOS (right)

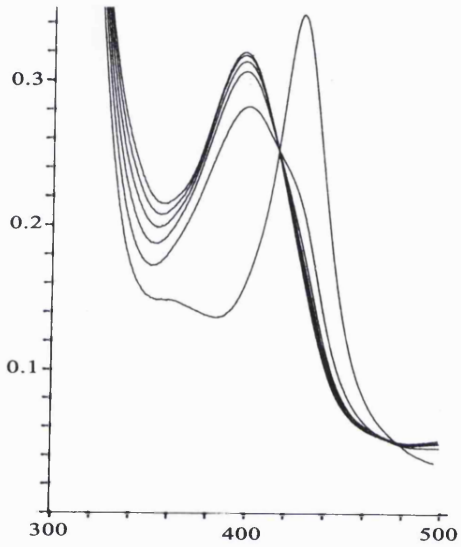


Kd=9.1 (±0.5) μM

Kd=6.6 (±0.3) μM

Figure 5.9 SEITU binding to SANOS (left) and iNOS (right)

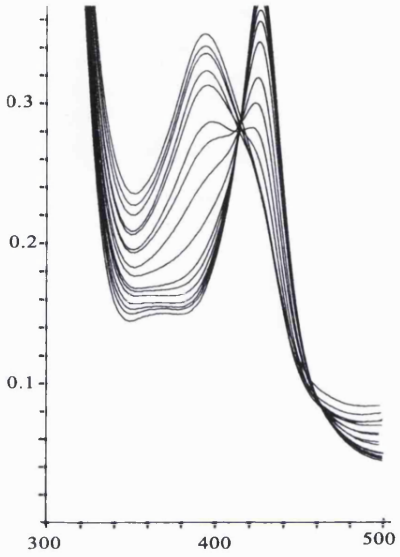


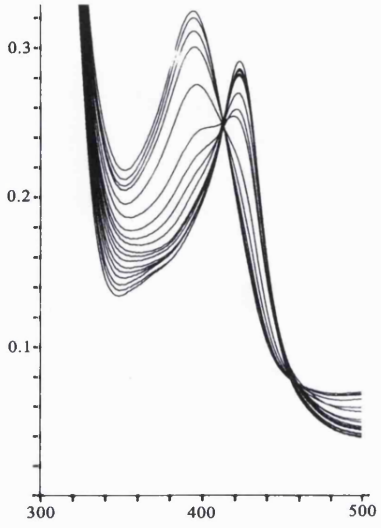


Kd=5.8 (±0.2) μM

Kd=0.4 (±0.1) μM

Figure 5.10 L-NIL binding to SANOS (left) and iNOS (right)





Kd=60.0 (±11.0) μM

Kd=9.4 (±1.0) μM

5.7. Hydrogen peroxide oxidation of NOHA by SANOS

N^G-hydroxy-L-arginine (NOHA) has been recognised as an NO synthetic intermediate by many scientists (Stuehr, D.J., *et al.*, 1991; Korth, H., *et al.*, 1994; Campos, K.L., *et al.*, 1995; Clague, M.J., *et al.*, 1997; Abu-Soud, H.M., *et al.*, 1997; Witteveen, C.F.B., *et al.*, 1998; Moali, C., *et al.*, 1998), and is formed after the first step of enzymatic NO generation.

To study the second step of NO formation, (Pufahl, R.A., *et al.,* 1995, Ghosh, D.K., *et al.,* 1997, & Rusche, K.M., *et al.,* 1998) various groups have carried out the hydrogen peroxide supported oxidation of N^G-L-arginine by NOS to form L-citrulline and nitrite/nitrate.

The Ghosh group used the murine iNOS heme domain to convert NOHA to L-citrulline and NO in an H_2O_2-supported reaction.

The method used in this thesis was a modified version of the Ghosh protocol and is described in 2.10.3. **Figures 5. 11 & 5.12** show the two proteins (iNOS and SANOS) used in the study, and **Table 5.2** shows the results of the reactions.

Murine iNOS heme domain can convert more than 100 mM/mg/protein NOHA to nitrite in the presence of SOD and H_2O_2. As a control, the NOS inhibitor SEITU was used to demonstrate that the reaction was specific to the NOS active site, and not simply a consequence of non-enzymatic heme-mediated conversion. The other two inhibitors used in the assays, NA and L-NMMA, only partly inhibited the conversion. BH_4 appears to play a crucial role in the iNOS-mediated conversion of NOHA to NO, and without it the conversion almost stopped. There is no detectable NO product without NOHA, suggesting that the reaction is very specific for NOHA as substrate. The SANOS-mediated conversion of NOHA to NO is less efficient than that shown by the iNOS heme domain. The amount of SANOS protein used in the experiments was 4-fold greater than the iNOS heme domain; so overall the actual activity is one fiftieth of the murine iNOS heme domain. All the reactions tested demonstrated a

Figure 5.11 Murine iNOS heme domain H_2O_2 shunt assay

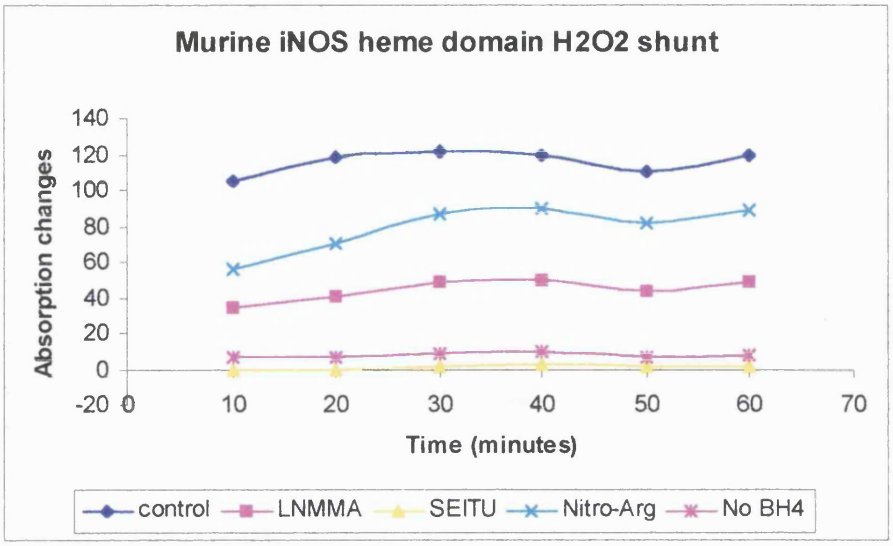


Figure 5.12 SANOS H_2O_2 shunt assay

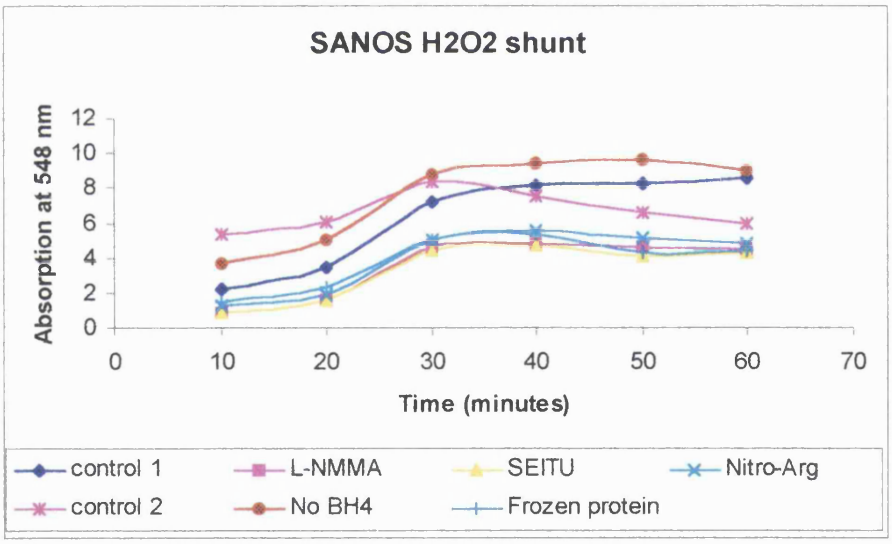


Table 5.1 Compound binding to SANOS, murine iNOS and rat nNOS

Compound	SANOS Ki (μM)	Murine iNOS Ki (μM)	Rat nNOS Ki (μM)
Imidazole	235.3	81.5	133
L-Arg	15.6	19.3	2.9
N-hydroxyarginine	3.2	4.7	1.7
Nitroarginine	7.5	4.9	0.81
S-EITU	5.7	0.39	0.13
L-NMMA	9.1	5.4	0.48
1400W	9.1	6.4	3.3
L-NIL	60.0	9.4	3.6

Table 5.2 H_2O_2 shunt result

iNOS	10 min	20min	30min	40min	50min	60min
Control	105.35	117.97	121.56	118.98	110.40	119.12
L-NMMA	33.89	40.52	48.70	49.67	43.94	48.36
SEITU	0	0	1.68	2.23	1.22	2.00
Nitro-Arg	55.76	70.74	86.26	89.61	81.70	88.88
-BH_4	6.38	6.60	8.51	8.54	6.80	7.30
-NOHA	0	0	0	0	0	0
SANOS						
Control	2.17	3.49	7.22	8.15	8.28	8.53
LNMMA	0.82	1.52	4.57	4.83	4.64	4.49
SEITU	0.85	1.57	4.38	4.68	4.09	4.24
Nitro-Arg	1.26	1.90	5.06	5.55	5.15	4.79
Controlx2	5.34	6.10	8.33	7.47	6.57	6.00
-BH_4	3.68	5.04	8.76	9.39	9.58	8.94
+Arginine	0	0	0	0	0	0
Frozen	1.43	2.33	5.00	5.28	4.29	4.40
-NOHA	0	0	0	0	0	0
-Protein	0	0	0	0	0	0
-H_2O_2	0	0	0	0	0	0

Time-dependent pattern. Control-2 had twice the amount of protein than control-1. Overall, three NOS inhibitors, L-NMMA, SEITU, and NA were able to decrease the formation of NO. As predicted from the previous BH_4 binding experiments, the generation of NO by SANOS with NOHA is not influenced by BH_4 (**Table 5.2**).

5.8. Summary

The characterisation of SANOS was carried out with a series of experiments involving substrate and inhibitor binding, and by the hydrogen peroxide supported oxidation of NOHA by SANOS using optical difference spectrophotometry.

The results supported the hypothesis that bacterial-derived SANOS is very similar in its enzymology to the equivalent domain from mammalian NOS. In the substrate binding experiments, SANOS exhibited a biochemical profile similar to that of the iNOS heme domain. The largest difference in binding was with the inhibitor SEITU. SEITU is a non-amino acid competitive inhibitor of NOS, and has been co-crystallised with human eNOS and iNOS heme domains (Fischmann, T.O., *et al.*, 1999), and also with SANOS (Appendix 1).

Mammalian NOS heme domains are capable of converting the arginine analogue NOHA into NO in the presence of H_2O_2. SANOS can catalyse the same reaction. This oxidise activity was independent of BH_4 and was inhibited by NOS inhibitors.

CHAPTER 6

IDENTIFICATION, CLONING AND EXPRESSION OF STAPHRED, A *S.aureus* PROTEIN WITH HOMOLOGY TO THE C-TERMINAL DOMAIN OF MAMMALIAN NOS

CONTENTS

6.1. Introduction

Research on the biochemistry of NOS (reviewed in Titheradge, M.A. 1998) has demonstrated that the protein can be divided into two independently functional domains an N-terminal oxygenase (heme) domain, and a C-terminal reductase domain. The C-terminal domain of NOS can catalyse an NADPH-dependent reduction of cytochrome c, whereas the N-terminal fragment retains an absorption spectrum characteristic of a heme containing protein. The two domains of the protein were conveniently generated by treatment with a protease, allowing the isolation of two fragments retaining ligand binding and catalytic activities corresponding to parts of the overall NOS reaction.

In 1996, Chen *et al.* (Chen, P.F., *et al.*, 1996) reconstituted NOS activity by mixing the two domains of eNOS. In their work, the N-and C-terminal eNOS domains were expressed in the baculovirus/insect cell system and purified independently. Although the overall NOS activity of the mixed domains was one-twentieth of the original enzyme, it suggested that the activity reconstitution by separate domains was possible. In 1997 and 1998, Choi *et al.* published their work on the measurement of NOS activity in *S.aureus* and its culture medium (Choi, W.S., *et al.*, 1997,1998) using the radioactive citrulline assay. These authors also showed a protein band in extracts of *S.aureus* that cross-reacted with anti murine iNOS antibody. The reported NOS signal was weak, and suggested that the protein recognised had a molecular weight of 55 Kda. While these reports suggested that a bacterial NOS-like protein might exist, further molecular support in the form of the identification of a gene was missing.

In previous chapters the cloning and characterisation of a gene encoding a protein with extensive homology to the N-terminal domain of NOS has been described. This chapter describes the cloning and expression of a *S.aureus* protein with extensive homology to the NOS reductase domain, STAPHRED. The objective of this part of the project was to attempt reconstitution experiments for NOS by mixing the two separate NOS-like domains (as has been described for mammalian NOS) and attempting to regenerate NO synthase activity. An alternative strategy relied on the

co-expression of SANOS and STAPHRED in the same *E. coli* background, and attempting to measure NOS activity.

6.2. STAPHRED in *S.aureus*

Using BLAST, a protein was found in the unfinished genomic DNA sequence of *S.aureus* with a high degree of similarity to the NOS reductase domain (NADPH cytochrome P450 reductase, NCPR). **Appendix 3** shows the BLAST result. The DNA sequence is capable of encoding a protein of 628 amino acids, with a calculated Mr of 73 kDa.

When STAPHRED was aligned with NCPR (**Figure 6.1**), the similarity between the two proteins was 55%. Additionally the two proteins comprise a similar number of amino acid residues: STAPHRED has 628 amino acids, while NCPR has 677 amino acids.

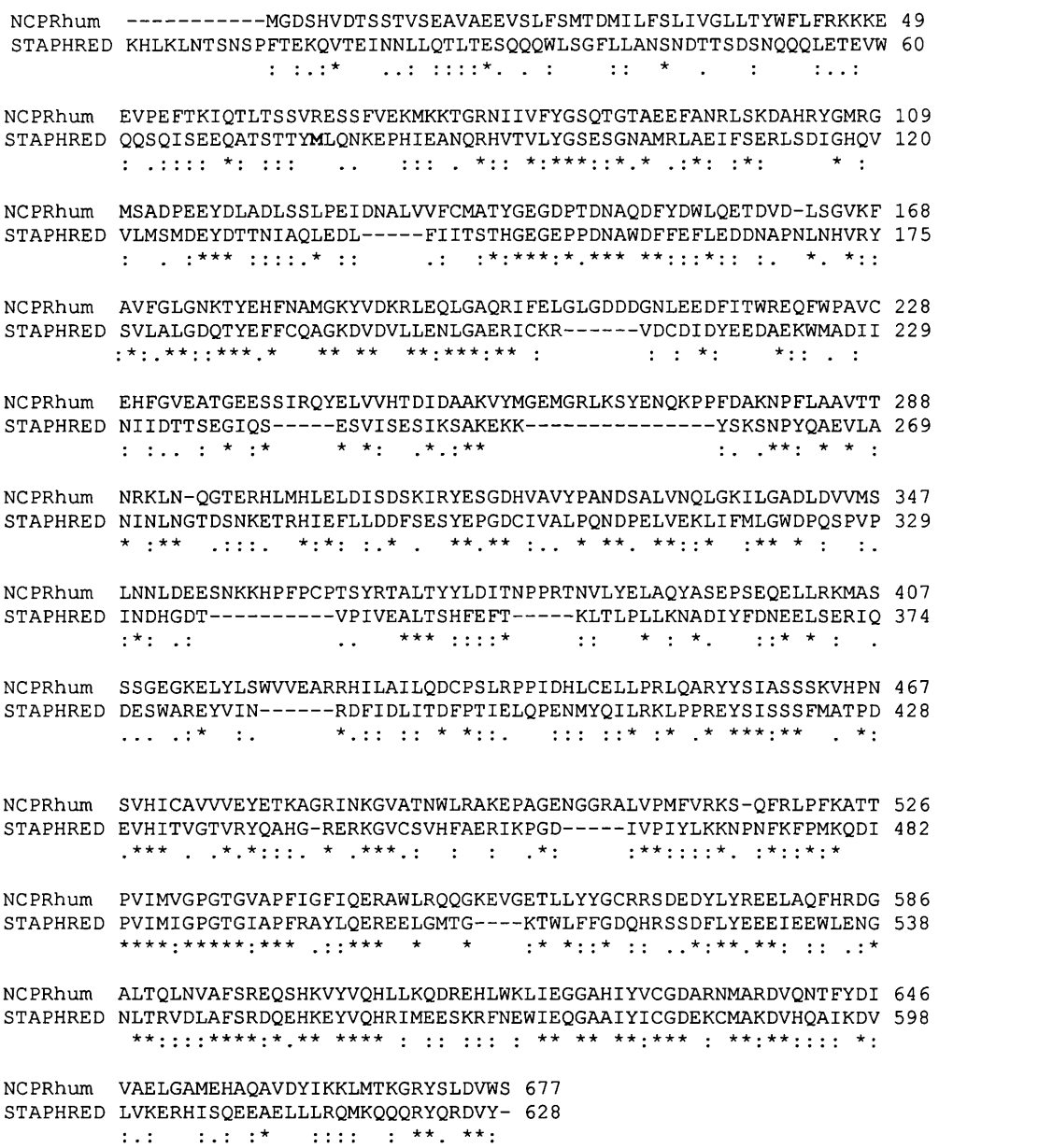
Figure 6.2 shows the alignment of STAPHRED and the rat nNOS reductase domain. The similarity between the two proteins is around 50%. The crystal structure of rat liver NCPR was published in 1997 (Wang, M., *et al.*, 1997). **Figure 6.3** shows the motif alignment according to the structure proposed by Wang.

Importantly, the sequences for motifs involved in FAD, FMN and NADPH binding are considerably conserved, and these elements are also conserved in the C-terminal NOS domain.

6.3. PCR amplification, molecular cloning and DNA sequence of STAPHRED

A pair of primers was synthesised according to the DNA sequence of the gene encoding STAPHRED from BLAST.

Figure 6.1 Alignment of NCPR and STAPHRED



***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Figure 6.2 Alignment of STAPHRED and reductase domain of rat nNOS

```
NOS       -KTLCEIFKHAFDAKAMSMEEYDIVHLEHEALVLVVTSTFGNGD---PPENGEKFGCALM 56
STAPHRED KHLKLNTSNSPFTEKQVTEINNLLQTLTESQQQWLSGFLLANSNDTTSDSNQQQLETEVW 60
          :   :  : .*  * ::  :  :  * ..    :    :.*.:   . .* :::   :

NOS       EMRHPNSVQEERKSYKVRFNSVSSYSDSRKS-----SGDGPDLRDNFESTGPLANVRFSV 111
STAPHRED QQSQISEEQATSTTYMLQNKEPHIEANQRHVTVLYGSESGNAMRLAEIFSERLSDIGHQV 120
         :  : .. *   .:* :: :.    ::.*:      * .*  :*     :  *::: ..*

NOS       FGLGSRAYPHFCAFGHAVDTLLEELGGE--------RILKMREGDELCGQEEAFRTWAKK 163
STAPHRED VLMSMDEYDTTNIAQLEDLFIITSTHGEGEPPDNAWDFFEFLE-DDNAPNLNHVRYSVLA 179
         . :.   *            :: .  **         :::: * *: . : : .*  .

NOS       VFKAACDVFCVG-DDVNIEKPNNSLISNDRSWKRNKFRLTYVAEAPDLTQGLSNVHKKRV 222
STAPHRED LGDQTYEFFCQAGKDVDVLLEN---LGAERICKRVDCDIDYEEDAEKWMADIINIIDT-- 234
         : . : :.** . .**::   *   :. :*  ** .  : *  :* .   .: *: ..

NOS       SAARLLSRQNLQSPKFSRSTIFVRLHTNGNQELQYQPGDHLGVFPGNHEDLVNALIERLE 282
STAPHRED -TSEGIQSESVISESIKSAKEKKYSKSNPYQAEVLANINLNGTDSNKETRHIEFLLDDFS 293
          ::. :. :.: * .:. :.     ::*  *       :  *. ..:.   :: *:: :.

NOS       DAP-PANHVVKVEMLE-ERNTALGVISNWKDESRLPP------CTIFQAFKYYLDITTPP 334
STAPHRED ESYEPGDCIVALPQNDPELVEKLIFMLGWDPQSPVPINDHGDTVPIVEALTSHFEFTKLT 353
         ::  *.: :* :   : *    * .: .*. :* :*        .*.:*:. ::::*. .

NOS       TPLQLQQFASLATNEKEKQRLLVLSKGLQEYEEWKWGKNPTMVEVLEEFPSIQMPATLLL 394
STAPHRED LPLLKNADIYFDNEE-------LSERIQDESWAREYVINRDFIDLITDFPTIELQPENMY 406
          **  :    : .:*       : .:  :*    ::  *  ::::: :**:*:: .  :

NOS       TQLSLLQPRYYSISSSPDMYPDEVHLTVAIVSYHTRDGEGPVHHGVCSS-WLNRIQADDV 453
STAPHRED QILRKLPPREYSISSSFMATPDEVHITVGTVRYQAHGRE---RKGVCSVHFAERIKPGDI 463
           *  * ** ******    *****:**. * *:::. *   ::****  : :**:..*:

NOS       VPCFVRGAPSFHLPRNPQVPCILVGPGTGIAPFRSFWQQRQFDIQHKGMNPCPMVLVFGC 513
STAPHRED VPIYLKKNPNFKFPMKQDIPVIMIGPGTGIAPFRAYLQER----EELGMT-GKTWLFFGD 518
         ** :::  *.*::* : ::* *::**********:: *:*    :. **.     *.**

NOS       RQSKIDHIYREETLQAKNKGVFRELYTAYSREPDRPKKYVQDVLQEQLAESVYRALKEQG 573
STAPHRED QHRSSDFLYEEEIEEWLENGNLTRVDLAFSRDQE-HKEYVQHRIMEESKR--FNEWIEQG 575
         :: . *.:*.**  :  ::* : .:  *:**: :  *:***. : *:  .  :.   ***

NOS       GHIYVCG-DVTMAADVLKAIQRIMTQQGKLSEEDAGVFISRLRDDNRYHEDIFGVTLRTY 632
STAPHRED AAIYICGDEKCMAKDVHQAIKDVLVKERHISQEEAELLLRQMKQQQRYQRDVY------- 628
         . **:** :  ** ** :**: ::.:: ::*:*:* ::: ::::::**:.*::

NOS       EVTNRLRSESIAFIEESKKDADEVFSS 659
STAPHRED ---------------------------
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Figure 6.3 Motif alignment of NOS, cytochrome P450 reductase and STAPHRED

			Phosphate moiety		FMN ring (re-face)		FMN ring (si-face)
FMN	NOS	755	ATILYATETGKSQAYAKTLC	803	LVVTSTFGNGDPPENGEKFGCAL	875	ANVRFSVFGLGSRAYPHFCAFGHAVDTLL
binding	NCPR	80	IIVFYGSQTGTAEEFANRLS	134	VFCMATYGEGDPTDNAQDFYDWL	164	SGVKFAVFGLGNKTYEHFNAMGKYVDKRL
regions	STAPHRED	91	VTVLYGSESGNAMRLAEIFS	140	FIITSTHGEGEPPDNAWDFFEFL	171	NHVRYSVLALGDQTYEFFCQAGKDVDVLL

			FAD ring (si-face)		Adenine		pyrophosphate		FAD ring (re-face)
FAD	NOS	1170	LQPRYYSISSSPDMYPDEVH	1190	LTVAIVSYHTRDGE	1204	GPVHHGVCSSWLN-RI	1389	RYHEDIFGV
Binding	NCPR	451	LQARYYSIASSSKVHPNSVH	471	ICAVVVEYETKAGR	485	..INKGVATNWLRAKE	670	RYSLDVWS
Regions	STAPHRED	412	LPPREYSISSSFMATPDEVH	432	ITVGTVRY-QAHGR	445	..INKGVATNWLRAKE	622	RYQRDVY

			Pyrophosphate		NADPH adenine
NADPH	NOS	1244	CILVGPGTGIAPFRSFWQQR	1318	RPKKYVQDVLQEQLAESVYRALKEQGGHIYVCGDVT-MAADV
Binding	NCPR	528	VIMVGPGTGVAPFIGFIQER	600	SHKVYVQHLLK-QDREHLWKLI-EGGAHIYVCGDAN-MARDV
Regions	STAPHRED	484	VIMIGPGTGIAPFRAYLQER	552	EHKEYVQHRIME-ESKRFNEWI-EQGAAIYICGDEKCMAKDV

The primers are:

5' primer 5'-CCGCGTCGACCGAAACATTTGAAATTAAATACATCTAATAG-3'

3' primer 5'-GGTCGACCTAATAAACATCACGTTGATAGCGTTG-3'

In order to facilitate cloning, a *SalI* site was designed into both ends of the primers for STAPHRED cloning. The PCR method was as described in section 2.7.12, and the product was cloned into the TOPO vector. Determination of the STAPHRED DNA sequence was as described (2.7.13). **Figure 6.4** shows the DNA and peptide sequences of STAPHRED respectively.

6.4. Expression of STAPHRED in *E. coli*

After the STAPHRED DNA sequence had been verified, it was cloned into pET28a. The recombinant plasmid was subsequently transformed into *E. coli* BL21 (DE)3 for expression. **Figure 6.5** shows the construction of the expression recombinant plasmid of STAPHRED in pET28a. **Figure 6.6** shows a SDS-PAGE gel stained with Coomassie blue and Western blot with anti-multihistidine antibody. The expression level of STAPHRED is quite low in *E. coli*, and it is difficult to distinguish a STAPHRED-specific band by Coomassie blue staining in the time course. However, a STAPHRED-specific band can be identified by Western blotting using the anti-multihistidine antibody.

6.5. Purification of STAPHRED

TALON metal affinity resin was used to purify the expressed recombinant STAPHRED protein. **Figure 6.7** shows the purification process. STAPHRED comprises 628 amino acids with a calculated Mr of 73 kDa. After eluting from the TALON column, some extra proteins appear associated with STAPHRED.

Figure 6.4 The DNA and peptide sequences of STAPHRED

```
            AAA CAT TTG AAA TTA AAT ACA TCT AAT AGT CCA TTT
              K   H   L   K   L   N   T   S   N   S   P   F    12

ACA GAA AAA CAA GTC ACA GAG ATT AAC AAT CTG CTG CAA ACA TTA
 T   E   K   Q   V   T   E   I   N   N   L   L   Q   T   L   27

ACA GAG AGC CAA CAA CAG TGG CTT AGT GGT TTT TTA CTA GCT AAT
 T   E   S   Q   Q   Q   W   L   S   G   F   L   L   A   N   42

AGT AAC GAT ACG ACA AGT GAT AGT AAT CAA CAA CAA TTA GAG ACA
 S   N   D   T   T   S   D   S   N   Q   Q   Q   L   E   T   57

GAA GTG TGG CAA CAA TCA CAA ATA TCA GAA GAA CAA GCA ACT TCA
 E   V   W   Q   Q   S   Q   I   S   E   E   Q   A   T   S   72

ACA ACG TAT ATG TTA CAA AAT AAA GAG CCA CAT ATC GAA GCT AAT
 T   T   Y   M   L   Q   N   K   E   P   H   I   E   A   N   87

CAG CGG CAT GTT ACA GTG CTA TAT GGT TCT GAA TCA GGT AAT GCC
 Q   R   H   V   T   V   L   Y   G   S   E   S   G   N   A  102

ATG CGT TTA GCT GAA ATT TTT TCA GAA CGT TTA AGT GAT ATC GGA
 M   R   L   A   E   I   F   S   E   R   L   S   D   I   G  117

CAT CAA GTT GTT TTG ATG TCA ATG GAT GAA TAT GAT ACG ACA AAC
 H   Q   V   V   L   M   S   M   D   E   Y   D   T   T   N  132

ATC GCG CAG TTA GAA GAT TTA TTT ATT ATT ACG TCT ACT CAT GGT
 I   A   Q   L   E   D   L   F   I   I   T   S   T   H   G  147

GAA GGA GAA CCG CCT GAT AAT GCA TGG GAT TTC TTT GAA TTT TTA
 E   G   E   P   P   D   N   A   W   D   F   F   E   F   L  162

GAA GAC GAT AAC GCA CCT AAT TTA AAT CAT GTG AGA TAT TCA GTA
 E   D   D   N   A   P   N   L   N   H   V   R   Y   S   V  177

CTA GCT TTA GGT GAT CAA ACA TAT GAA TTT TTC TGT CAA GCC GGT
 L   A   L   G   D   Q   T   Y   E   F   F   C   Q   A   G  192

AAA GAT GTA GAT GTT TTA CTA GAA AAT CTA GGC GCT GAG CGT ATA
 K   D   V   D   V   L   L   E   N   L   G   A   E   R   I  207

TGT AAG CGT GTA GAT TGT GAT ATT GAT TAT GAA GAA GAC GCA GAA
 C   K   R   V   D   C   D   I   D   Y   E   E   D   A   E  222

AAG TGG ATG GCA GAC ATC ATT AAT ATT ATT GAT ACC ACA TCA GAA
 K   W   M   A   D   I   I   N   I   I   D   T   T   S   E  237

GGT ATT CAA AGT GAA TCG GTG ATA AGT GAA TCA ATT AAG TCT GCC
 G   I   Q   S   E   S   V   I   S   E   S   I   K   S   A  252

AAA GAA AAG AAA TAT TCT AAA TCA AAT CCA TAC CAA GCA GAA GTA
 K   E   K   K   Y   S   K   S   N   P   Y   Q   A   E   V  267

TTA GCG AAT ATC AAT TTA AAT GGT ACC GAT TCA AAT AAA GAA ACA
 L   A   N   I   N   L   N   G   T   D   S   N   K   E   T  282

CGA CAT ATA GAA TTT TTA CTT GAT GAT TTT AGT GAA TCA TAT GAA
 R   H   I   E   F   L   L   D   D   F   S   E   S   Y   E  297

CCA GGA GAT TGT ATA GTA GCA TTA CCG CAA AAC GAC CCT GAA TTG
 P   G   D   C   I   V   A   L   P   Q   N   D   P   E   L  312

GTT GAA AAA CTA ATA TTC ATG TTA GGT TGG GAT CCG CAA TCT CCG
 V   E   K   L   I   F   M   L   G   W   D   P   Q   S   P  327

GTG CCA ATT AAT GAT CAT GGT GAT ACA GTT CCT ATT GTT GAA GCA
 V   P   I   N   D   H   G   D   T   V   P   I   V   E   A  342

CTA ACA TCA CAT TTT GAA TTT ACT AAA TTA ACA TTG CCA TTA TTG
 L   T   S   H   F   E   F   T   K   L   T   L   P   L   L  357
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AAA AAT GCA GAT ATC TAT TTT GAC AAT GAA GAA TTA TCT GAA CGT
 K   N   A   D   I   Y   F   D   N   E   E   L   S   E   R  372

ATT CAA GAT GAG TCA TGG GCG CGT GAA TAT GTT ATA AAT CGG GAC
 I   Q   D   E   S   W   A   R   E   Y   V   I   N   R   D  387

TTT ATA GAT TTA ATA ACA GAT TTT CCA ACT ATA GAA TTA CAA CCT
 F   I   D   L   I   T   D   F   P   T   I   E   L   Q   P  402

GAG AAT ATG TAT CAA ATC CTT AGA AAA TTA CCA CCA AGA GAG TAT
 E   N   M   Y   Q   I   L   R   K   L   P   P   R   E   Y  417

TCG ATT TCT AGT AGT TTT ATG GCA ACG CCA GAT GAA GTG CAT ATT
 S   I   S   S   S   F   M   A   T   P   D   E   V   H   I  432

ACC GTT GGT ACG GTT CGT TAT CAA GCA CAT GGA CGT GAG AGA AAA
 T   V   G   T   V   R   Y   Q   A   H   G   R   E   R   K  447

GGT GTA TGC TCG GTT CAT TTT GCT GAG CGA ATT AAA CCA GGC GAT
 G   V   C   S   V   H   F   A   E   R   I   K   P   G   D  462

ATA GTA CCA ATT TAT TTG AAG AAA AAT CCG AAC TTC AAA TTT CCG
 I   V   P   I   Y   L   K   K   N   P   N   F   K   F   P  477

ATG AAG CAA GAT ATA CCG GTT ATT ATG ATT GGA CCA GGT ACT GGA
 M   K   Q   D   I   P   V   I   M   I   G   P   G   T   G  492

ATT GCT CCT TTT AGA GCA TAT TTA CAA GAA CGT GAA GAA CTT GGT
 I   A   P   F   R   A   Y   L   Q   E   R   E   E   L   G  507

ATG ACT GGA AAA ACA TGG TTG TTC TTT GGT GAT CAA CAC CGT AGT
 M   T   G   K   T   W   L   F   F   G   D   Q   H   R   S  522

TCT GAC TTT TTA TAT GAA GAA GAA ATA GAA GAA TGG CTT GAA AAT
 S   D   F   L   Y   E   E   E   I   E   E   W   L   E   N  537

GGA AAC TTA ACA CGC GTA GAT TTA GCA TTT TCA AGA GAC CAA GAA
 G   N   L   T   R   V   D   L   A   F   S   R   D   Q   E  552

CAC AAA GAA TAT GTA CAG CAT CGT ATA ATG GAA GAA AGT AAA CGT
 H   K   E   Y   V   Q   H   R   I   M   E   E   S   K   R  567

TTC AAT GAA TGG ATT GAG CAA GGC GCA GCA ATC TAT ATT TGT GGC
 F   N   E   W   I   E   Q   G   A   A   I   Y   I   C   G  582

GAT GAA AAA TGT ATG GCG AAA GAT GTC CAT CAA GCC ATT AAA GAT
 D   E   K   C   M   A   K   D   V   H   Q   A   I   K   D  597

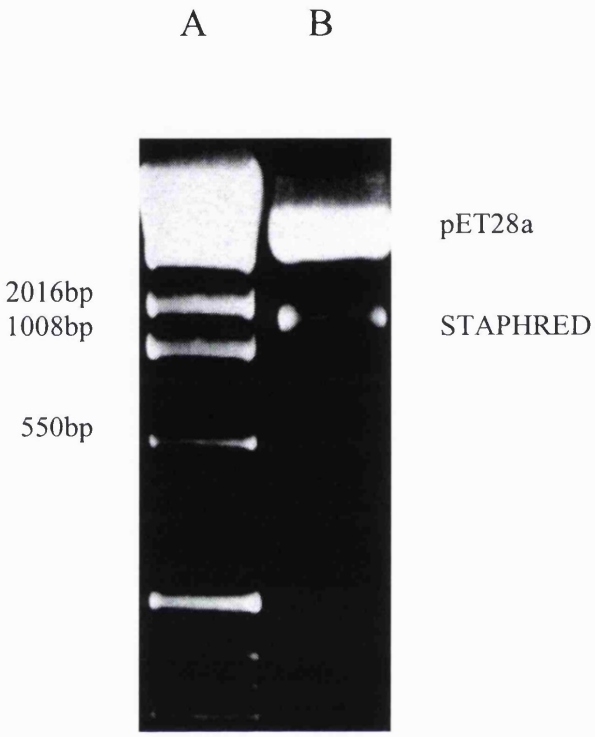
GTA TTG GTA AAA GAA CGT CAT ATT TCT CAA GAA GAA GCA GAG TTA
 V   L   V   K   E   R   H   I   S   Q   E   E   A   E   L  612

TTA TTG CGA CAA ATG AAA CAA CAA CAA CGC TAT CAA CGT GAT GTT
 L   L   R   Q   M   K   Q   Q   Q   R   Y   Q   R   D   V  627

TAT TAA
 Y   *      628
```

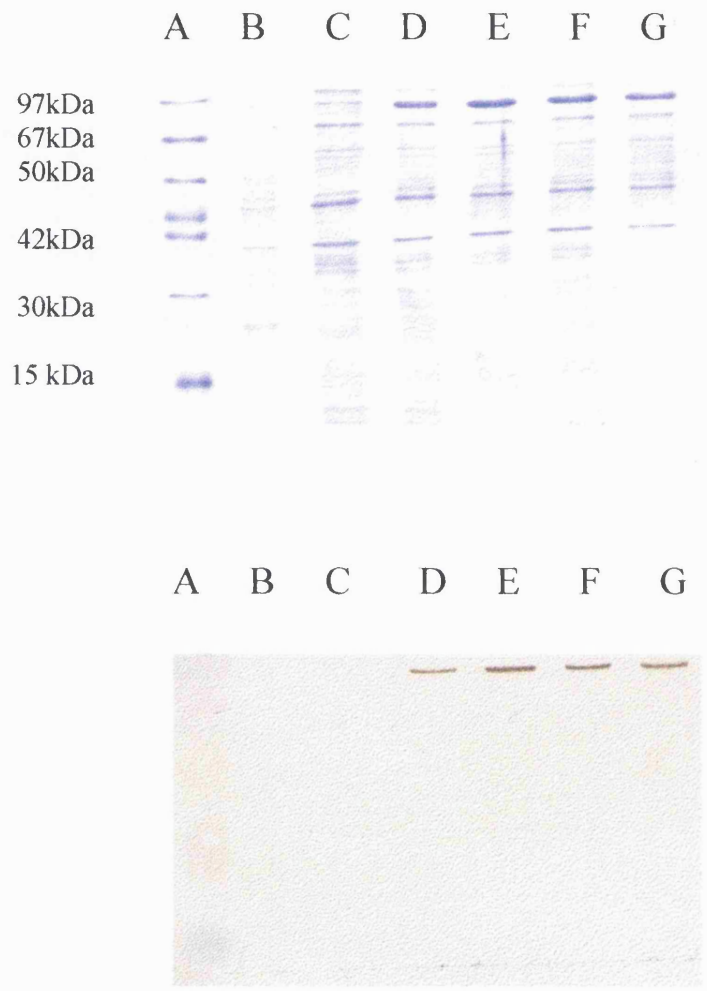
Bold/underlined residues indicate the possible starting codons.

Figure 6.5 Cloning of STAPHRED in pET28a



A. DNA marker; B. cloning of STAPHRED in pET28a.

Figure 6.6 STAPHRED expressed in *E.coli*

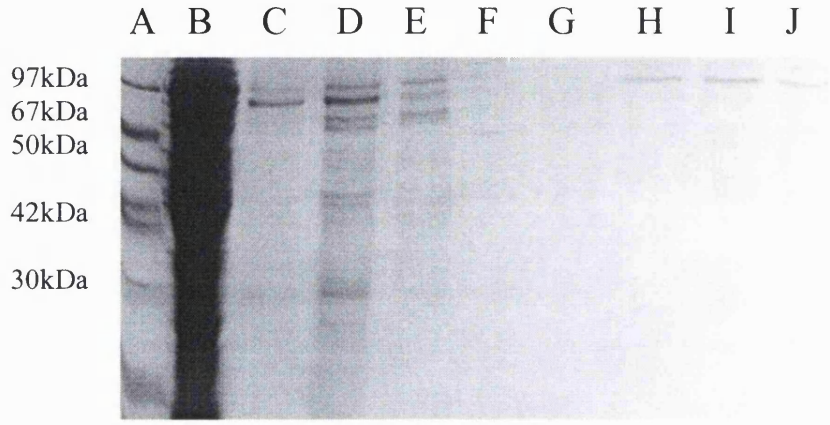


Up. Coomassie blue staining;

Below. Western blot.

A. Protein marker; B. pET28a induction; C. STAPHRED in *E.coli* uninduced; D to G. STAPHRED in *E.coli* induction from 0.5 to 3 hours.

Figure 6.7 STAPHRED purification



A. Marker; B. supernatant of cell lysate; C. flow through of the supernatant; D and E. 10 mM imidazole wash;
F to J. 1st to 5th elution.

6.6. Co-expression SANOS and STAPHRED

Following the successful expression of STAPHRED in *E. coli*, experiments were designed to co-express SANOS and STAPHRED in the same *E. coli* background. To overcome potential incompatibility problems with the expression plasmids, pACYC184 was used to clone STAPHRED, while SANOS protein was to be expressed in a pUC-based replicon.

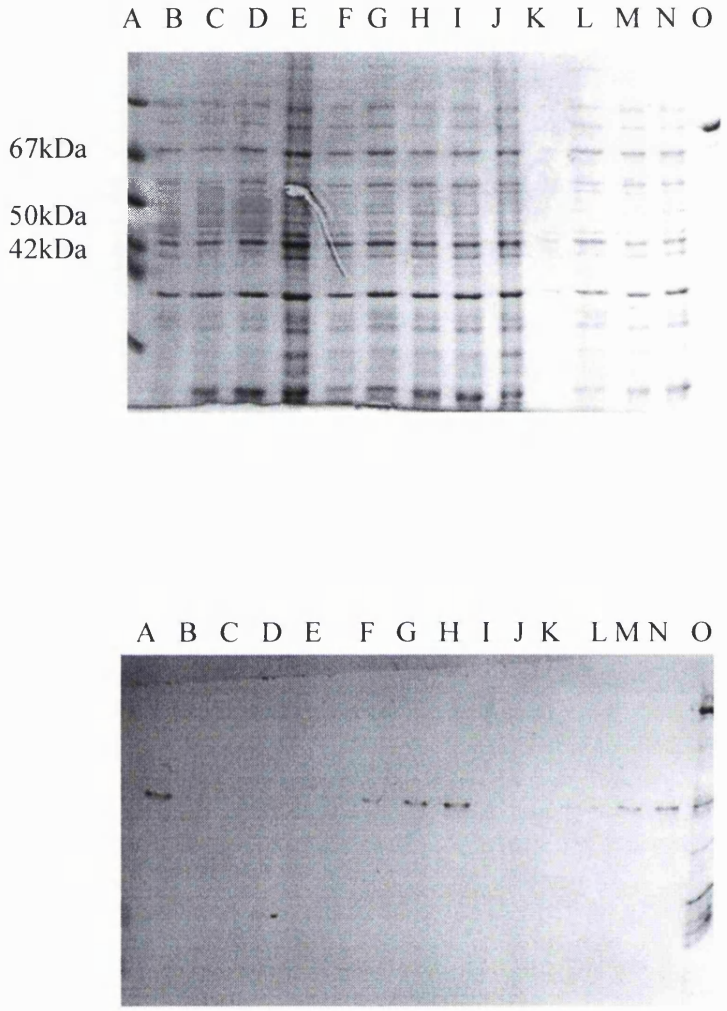
As STAPHRED was originally expressed in pET28a, then the T7 promoter, terminator and *lacI* genes were retained in the cloned fragment for optimal expression in pACYC184. The recombinant plasmid with STAPHRED in pET28a was cut with *SmaI* and *FspI*. Plasmid pACYC184 was cut by *EcoRV* and ligated to the larger fragment from pET28a containing the STAPHRED fragment and control regions. The new recombinant plasmid was named pACSARED. The two recombinant plasmids, pACSARED and SANOS (in pCWori), were co-transformed into *E. coli* BL21(DE)3. **Figure 6.8** shows the expression results.

Interestingly, only an expressed SANOS band could be recognised by anti multi-histidine antibody. The presence of His-tagged STAPHRED could not be detected with the same antibody. This suggests that there is a problem with the expression levels of STAPHRED in the pACYC-background.

6.7. Summary

BLAST was used to identify a protein in *S.aureus* with considerable identity to the C-terminal domain of mammalian NOS and NADPH cytochrome P450 reductase. Sequence alignments suggested that the protein might be able to bind FAD, FMN and NADPH, as motifs for these co-factor binding sites are apparent in the sequence.

Figure 6.8 Co-expression of SANOS and STAPHRED in *E.coli*



Up. Coomassie blue staining; Bottom. Western blot.

A. protein marker; B. SANOS in *E.coli*; C and I. uninduced culture; D and J. 0.5 hour induction; E and K. 1 hour induction; F and L. 2 hours induction; G and M. 3 hours induction; H and N. 4 hours induction; O. purified STAPHRED.

PCR was used to clone the gene encoding this protein with similarity to the C-terminal region of NOS, and DNA sequencing confirmed that the cloned product had the same sequence as that deposited in the database. The STAPHRED protein was expressed in *E.col,* and a soluble protein could be purified. The incorporation of a 6-his tag onto the N-terminus of the protein allowed for rapid purification. However, protein yields were quite low (estimated at 100 μg/Litre induction culture).

The main aim for the cloning of the STAPHRED protein was to attempt to generatethe activity of full-length NOS from the two domains. The failure to generate increased NOS activity following the mixing of *S.aureus* SANOS and STAPHRED domains may have resulted from inactivity of the STAPHRED protein. Although the protein was purified in a soluble form, there was no other assay that could have been carried out to verify whether or not the protein was active. In order to address this problem, co-expression studies were carried out where the SANOS domain was expressed in the same *E. coli* host as the STAPHRED domain. Interestingly only SANOS could be detected in the dual expression system, suggesting that cloning into the lower copy number pACYC replicon had compromised expression.

CHAPTER 7

DISCUSSION AND CONCLUSION

CONTENTS.

7.1. Overview

The NOS family has been intensively studied since the 1980's. A large amount of work has been carried out on the role of NO in disease and health, and this has included research on NOS localisation, isolation, expression, structural characterisation, and catalytic mechanisms. These works have been carried out on NOS from many species (rat iNOS, Adachi, H., *et al.*, 1993; human iNOS, Charles, I.G., *et al.*, 1993; rat nNOS, Charles, I.G., *et al.*, 1993; bovine eNOS, Chen, P.F., *et al.*, 1996; human eNOS, Janssens, S.P., *et al.*, 1992; chicken iNOS, Lin, A.W., *et al.*, 1996; murine iNOS, Lyons, C.R., *et al.*, 1992; pig, eNOS, Zhang, J.L., *et al.*, 1996).

However, despite all these studies, there has been no definitive molecular biological evidence for a bacterial NOS isoform.

The purpose of this study was to find NOS-like protein(s) in bacteria, and characterise them. Recent papers have shown the presence of NOS activity in cell lysates and culture medium of *S.aureus* (Choi, W.S., *et al.*, 1997, 1998), however the radioactive citrulline assay used in these experiments is prone to background that may well be confused with NOS activity. Although these studies gave a clue about bacterial NOS, definitive molecular evidence in the form of an isolated gene was missing. The task for this research project was to concentrate on NOS characterisation in the Gram-positive bacteria, *S.aureus*, and to extend this characterisation to other organisms if possible.

In this study, a NOS-like protein with properties similar to the N-terminal heme domain of mammalian NOS was isolated and characterised from *S.aureus*, and the protein was crystallised as part of collaboration with Dr. Stammers at Oxford University. Additionally, attempts were made to characterise a similar NOS-like protein from *B. subtilis*. A protein with similarity to the C-terminal reductase domain of NOS was also identified in *S.aureus*, and was studied at the same time. This study provided the first evidence for expression of a NOS-like protein in bacteria.

The discovery of sequence homology to a known protein or family of proteins often provides the first clues about the function of a newly sequenced gene. As DNA and amino acid sequence databases continue to grow in size they become increasingly useful in the analysis of newly sequenced genes and proteins because of the greater chance of finding such homologies (Altschul, S.F., *et al.*, 1990).

Using BLAST, a protein of unknown function was found in the unfinished *S.aureus* genome using the human iNOS cDNA sequence as a template. The deduced protein sequence was compared to human iNOS (**Figure 4.1**) and other NOS sequences (**Appendix 7**). Because of the similarity to NOS proteins, the new protein was named SANOS.

Following identification from BLAST, the sequence was cloned from *S.aureus* genomic DNA using specific PCR primers. Following sequence verification, the gene was cloned into an *E. coli* expression vector, and the protein expressed and purified. Functional studies were carried out to assess the biological activity of the protein based on the similarity of the protein to NOS. In a similar fashion to the NOS heme domain, SANOS was found to be associated with a prosthetic heme group. The protein can also bind L-arginine specifically, and can oxidise the NOS intermediate substrate NOHA to NO in the presence of SOD and H_2O_2. According to the alignment (**Figure 4.1**), the open reading frame encoding SANOS starts after the sequence that in mammalian NOS isoforms is responsible for the metal centre responsible for co-ordination to BH_4 (Raman, C.S., *et al.*, 1998). The absence of this motif suggests that, unlike the mammalian isoforms, SANOS may not be able to bind BH_4.

The properties of SANOS appear to be very similar to those reported for the N-terminal heme domain of NOS. SANOS was crystallised at Oxford University after it was further purified at University of Newcastle. The crystallisation results indicated that SANOS is a dimeric heme protein that can bind NAD.

7.2. Searching and comparing of NOS-like proteins in bacteria

7.2.1. NOS Heme domain-like protein in bacteria

When BLAST was used to search for NOS-like sequences in the data bases of finished and unfinished bacterial genomes, a family of proteins with homology to the NOS heme domain was found in several Gram-positive bacteria (**Figure 4.4**). NOS-like proteins were found in the genomes of: *Bacillus anthracis* (Anthra), *Bacillus halodurans* (Bacha, BAB04542), *Bacillus subtilis* (YFLM, C69811), *Deinococcus radiodurans* (Radio, B75256) and *Staphylococcus aureus* (SANOS, BAB58076). When translated, the NOS-like genes all appear to be capable of encoding proteins with similar lengths: 325, 366, 336, 356, and 362 amino acids, respectively. All of the predicted proteins start after the NOS metal centre implicated in BH_4 binding (107-112 in HiNOS; Raman, C.S., *et al.*, 1998). In addition, the amino acid residues believed to be important in NOS dimerisation are also changed (G453, A456 in HiNOS; Cho, H.J., *et al.*, 1995). Sequence alignments with mammalian NOS isoforms suggest that the NOS-like proteins from these bacteria all end before the CaM binding site (amino acids 506-526) in HiNOS, **Figure 1.1**.

The results from sequence alignments suggest that although these bacterial NOS-like proteins have a high degree of similarity to mammalian NOS, they may not be able to bind BH_4 or CaM. Additionally, because of the lack of BH_4-binding sites (involved in the dimerisation process), the possibility exists that the bacterial domains may exist in a monomeric form as compared with the dimeric organisation of all other NOS isoforms. But the crystal structure of SANOS seems to make the monomer hypothesis unlikely to be. SANOS appears as a dimer in crystallisation status although without BH_4-binding site.

As shown in **Figure 3.3**, the well-characterised heme-binding domain of mammalian NOS isoforms is highly conserved with a putative motif in bacterial NOS isoforms. Amino acid Glu 376 in human iNOS (Glu 361 in human eNOS) has been confirmed to be the arginine-binding site by mutagenesis and crystallisation studies (Chen, P.F., *et al.*, 1997; Gachhui, R., *et al.*, 1997; Fischmann, T.O., *et al.*, 1999). In all of the

bacterial NOS heme domains, this Glu residue is conserved. For example, in the bacterial sequences the Glu residue is at amino acid 242 in Bacha, at 222 in Radio, at 230 in SANOS, and 216 in YFLM, respectively. These findings suggest that the bacterial NOS-like proteins may be able to bind heme and L-arginine, and may even have NOS heme domain enzyme activity.

A NOS heme domain-like protein was identified by BLAST from *S.aureus* in the 'Unfinished microbial genome DNA data base' (**Appendix 2**). A comparison with the heme domain of human iNOS shows an overall identity of 41% and a similarity of 58% when conservative substitutions are taken into account (**Figure 4.1**). At the time of writing this thesis, the whole genome DNA sequence of *S.aureus* had not been finished (However a recent paper has been published about the whole *S.aureus* genomic DNA sequenece). For convenience, the protein encoded by this gene was called **SANOS**. A comparison with human iNOS (**Figure 4.1**) shows that a motif defining heme binding extends from amino acids 193 to 205 in human iNOS is conserved in SANOS (amino acids 164-176; Lowe, P.N., *et al.*, 1996). In addition, the L-arginine binding motif (from amino acids 367-380) in human iNOS is almost identical to a motif (from amino acids 224-237) in SANOS. These findings strongly suggested that SANOS could be a heme protein with ability to bind arginine and confirmed by the different tests later.

The biochemistry, physiology and molecular biology of *B. subtilis* have been extensively studied over the past 40 years. In particular, *B. subtilis* has been used to study post-exponential phase phenomena such as sporulation and competence for DNA uptake. In addition, *B. subtilis* is also the best-characterised member of the Gram-positive bacteria. The whole genome DNA sequence was finished in 1997. A total of 4,214,810 bp comprises 4,100 protein-coding genes. Over 4,000 putative protein coding sequences (CDSs) have been identified, with an average size of 890 bp, covering 87% of the genome sequence. It was found that 78% of the genes started with ATG, 13% with TTG and 9% with GTG. The NOS-like gene *yflM* is one of the genes identified as having a GTG as starting codon. In *B. subtilis*, genes were classified using the BLAST2P software running against a composite protein databank

compound of SWISS-PROT, TREMBL and *B. subtilis* proteins. Using this approach it was possible to assign at least one significant counterpart with a known function to 58% of the *B. subtilis* proteins. Thus, for up to 42% of the gene products, the function cannot be predicted by similarity to proteins of known function. 4% of the proteins are similar only to other unknown proteins of *B. subtilis*; 12% are similar to unknown proteins from some other organism; and 26% of the proteins are not significantly similar to any other proteins in the databanks. This preliminary analysis should be interpreted with caution, because only ~1,200 gene functions (30%) have been experimentally identified in *B. subtilis*. The '*y*' prefix was assigned to genes to emphasise that the function of that gene has not been ascertained (2,853 '*y*' genes, representing 70% of the genome).

A peptide, encoded by the *yflM* gene, and classified in the 'detoxification group', was labelled as having similarity to nitric oxide synthase. When the protein encoded by *yflM* was aligned with SANOS (**Figure 3.1**), the identity between the two proteins was shown to be 53.5%, and a similarity of 75%, suggesting that the two peptides might possess similar properties. There is also a high degree of similarity when alignments are carried out with human iNOS (**Figure 3.2, 3.3**).

7.2.2. NOS reductase domain-like protein in *S.aureus*

While a protein with similarity to the NOS heme domain was characterised from *S.aureus*, for the bacteria to be able to generate a fully functional NOS it would require an additional protein with identity to the NOS C-terminal domain. Using cytochrome P450 reductase (NCPR) as a template in BLAST searches (NCPR contains the FAD, FMN, and NADPH binding sites also conserved in NOS), a peptide sequence with high identity was recognised in *S.aureus* 8325. As shown in **Appendix 3**, a 628 amino acid polypeptide was identified, and named STAPHRED. The deduced amino acid sequence was aligned with human NCPR (**Figure 6.1**), and the rat nNOS reductase domain (**Figure 6.2**).

Figure 6.3 shows the domains alignment between STAPHRED, rat nNOS reductase

and human NADPH cytochrome P450 reductase. STAPHRED has a similar size when compared to NCPR (628 amino acids compared to 647 amino acids). Not surprisingly, STAPHRED (with 628 amino acids) is also very similar to the reductase domains of nNOS, eNOS, and iNOS, (684, 693, 621 amino acids respectively). The length of these functional reductase domains varies, and bacterial cytochrome $P450_{BM-3}$ comprises 1049 amino acids (Ruettinger, R.T., *et al.*, 1989). Importantly, the FMN, FAD, and NADPH binding motifs are very similar between STAPHRED, NCPR and NOS, supporting the hypothesis that STAPHRED may be capable of electron transfer reactions.

Forms of the nNOS reductase domain that are missing a functional CaM binding site catalyse cytochrome C or $FeCN_6$ reduction at rates that exceed those of the intact CaM–bound reductase domain (Stuehr, D.J., 1997). It appears from these studies that the CaM domain of NOS is not required for the electron transfer to these acceptors, and might actually be autoinhibitory in the absence of bound calmodulin (Stuehr, D.J., 1997). There is no motif in STAPHRED that corresponds to a CaM binding site (**Figure 6.3**). If STAPHRED can function as an electron donor as part of two-component NOS in *S.aureus* to generate NO, it is unlikely that CaM plays any part in the reaction. This is perhaps not surprising, as CaM has only been characterised to date in eukaryotes.

7.3. Cloning, expressing and purifying NOS-like proteins

PCR was used to amplify fragments of genes encoding SANOS, STAPHRED and YFLM from genomic DNA of *S.aureus* and *B. subtilis*. To reduce mis-incorporation, *Pfu* DNA polymerase was used in the amplification reaction. The DNA sequences recovered from these reactions were verified after the PCR products were cloned into the TOPO cloning vector. The sequences generated by PCR were found to be identical with the sequences reported in the databases. (**Figure 3.5, 3.6**; **Figure 4.6**; **Figure 6.4**).

The murine iNOS heme domain was crystallised following expression of recombinant protein in *E. coli* using the vector pCWori (Crane, B.R., *et al.*, 1997, 1998). This vector has been successfully used for the generation of recombinant heme proteins in *E. coli*, consequently it was disappointing when the strategy failed to generate soluble *yflM*–encoded protein. Despite this failure, the pCWori vector was selected for expression trials to attempt to express recombinant SANOS in *E. coli.*

The *E. coli* expression vector pCWori has been used to express a variety of NOS isoforms during the past few years, such as: bovine eNOS (Martasek, P., *et al.*, 1996; Rodriguez-Crespo, I., *et al.*, 1996), mouse iNOS (Wu, C., *et al.*, 1996; Ghosh, D.K., *et al.*, 1997; Rusche, K.M., *et al.*, 1998; Presta, A., *et al.*, 1998), rat nNOS (McMillan, K., *et al.*, 1995; Roman, L.J., *et al.*, 1995; Gerber, N.C., *et al.*, 1995), human eNOS (Rodriguez-Crespo, I., *et al.*, 1996), and human liver iNOS (Gerber, N.C., *et al.*, 1997). In this study, pCWori was used to express SANOS in *E. coli.* The expression and the purification procedures were carried out in accordance with the method of Rodriguez-Crespo, I., *et al.*, (1996). Accordingly, the induced expression was undertaken in the presence of δ-aminolevulinic acid, (a precursor of heme; Rodriguez-Crespo, I., *et al.*, 1996). Following, growth, induction, and centrifugation, the cell pellet is dark brown in colour, consistent with the accumulation of a heme containing protein. An interesting observation on the expression of these cultures was that the OD_{600} of the 48 hours induced culture of SANOS, (and the human iNOS heme domain) reached OD600 = 23. This figure is much higher than that for many other proteins expressed in *E. coli* (Sambrook, J., *et al.*, 1989).

7.3.1. YFLM

yflM was the first gene cloned in this study, and attempts were made to express recombinant protein from this gene in *E. coli.* The first attempt to express the *yflM*-encoded protein utilised the T_7 expression vector pET28a. Using this system, protein expression was readily achieved, and high levels were produced in *E. coli.* The cell pellet and supernatant fractions were separated by centrifugation after sonication.

Unfortunately, only insoluble *yflM*-encoded protein could be detected in the cell pellet after SDS-PAGE and Coomassie staining.

Although much effort was made to attempt to improve solubility of the *yflM*-encoded protein in *E. coli*, using different vectors and different *E. coli* strains (and in combinations, described in chapter 3), the protein remained in the pellet fraction. When anti-*yflM* anti serum was applied to expression analysis of the protein in *E. coli* and *B. subtilis*, a small component of the overall yield of the expressed protein could be detected in the supernatant (soluble) fraction by Western blotting. The purification process failed to generate sufficient quantities of the protein to proceed for further functional assays. Additional attempts to improve solubility, using a panel of detergents also failed (chapter 3).

The final attempt to express soluble *yflM* was by fusion expression with GST. In an attempt to keep the best properties of the pCWori plasmid (which is one of the few plasmids to be used successfully for the expression of heme proteins) and GST, a fusion expression vector was constructed by inducing GST fragment from pGEX4T-2 into pCWori. The recombinant plasmid was named pCWGST. The recombinant plasmid was transformed into BL21 (DE3) and protein induction carried out in the presence of 1 mM IPTG. Unfortunately, under all the conditions tested, the YFLM/GST fusion protein was still in the insoluble pellet fraction (**Figure 3.9**).

Northern blotting was carried out in order to determine whether or not *yflM* was expressed in *B. subtilis*. The *yflM* PCR product was used in these experiments as probe, and the random primer labelling method was used to generate a ^{32}P-labelled probe. The results demonstrated that *yflM* was expressed in *B. subtilis*, and the message detected was of a similar size to that of the positive control *E. coli* expressed *yflM* gene (**Figure 3.10**).

In summary, *yflM* can be expressed readily in *E. coli*, (albeit as an insoluble protein product) and its expression can be detected in *B. subtilis* (**Figure 3.10, 11**). For detailed expression research, two anti-*yflM* anti-bodies were raised in rabbits against

two peptides. The first peptide was from amino acid 121 (S) to amino acid 140 (G), while the second was from amino acid 276 (H) to amino acid 295 (K). The antiserum samples were called *yflM*121 and *yflM*276.

Both antibodies were used to identify the pattern of *yflM* expression in *B. subtilis* and *E. coli.* Using this approach, two main bands appeared (**Figure 3.11**). One band at 40 kDa, corresponds to the full-length *yflM*–encoded protein, the other is between 25 to 30 kDa and is recognised by antiserum *yflM*276. These western blotting results suggest that either the protein may be degraded during the preparation of the lysates, or alternatively, some post-translational process may be cleaving the protein to a smaller product.

7.3.2. SANOS

The expression of SANOS was detected in Northern blots in *S.aureus* and *E. coli* samples, using a SANOS PCR product as probe. The random primer labelling method was used to generate a ^{32}P-labelled probe. The results are shown in **Figure 4.7**, and demonstrate that a SANOS transcript can be detected at around the same size in both *S.aureus* and *E. coli.* (**Figure 3.10**).

Recombinant SANOS was purified by TALON affinity chromatography in accordance with the methods provided by CLONTECH. The supernatant of lysates of cultures of expressed SANOS was dark brown in colour. The SANOS protein was purified by TALON column chromatography (**Figure 4.8**). Following purification, the recombinant protein could be stored at a concentration of 10 mg/ml -80°C after ammonium sulphate precipitation. The protein could be used for functional assays, and for crystallisation studies (Appendix 4).

7.3.3. STAPHRED

The PCR amplification and DNA sequencing of *staphred*, (the gene encoding STAPHRED - the protein with identity to the C-terminal domain of NOS) from

S.aureus genomic DNA was carried out successfully. The sequence of the cloned gene was identical to the sequence in the database. The *staphred* gene was subcloned into pET28a for expression studies. Various experiments were carried out to analyse the expression of STAPHRED, however, the expression level was too low to be measured by Coomassie blue staining after SDS-PAGE. Soluble protein can be detected using antihistidine antibody (**Figure 6.6**). Recombinant STAPHRED can be purified by FPLC, but the low yield makes it difficult to use for further assays.

7.4. Characterisation of SANOS

Recombinant SANOS was used for all the following experiments. The first characteristic to be measured in SANOS was the presence of the prosthetic heme group. **Figure 4.9** shows the spectrum of SANOS compared with iNOS, recorded on a spectrophotometer (**Figure 4.10**). The spectrum shows that SANOS possesses the characteristic heme peaks at 420, 524, and 556 nm, with a very similar profile compared with the murine iNOS heme spectrum (Klatt, P., *et al.*, 1996). Optical difference spectrophotometry has been used as a probe of rat nNOS heme and substrate interaction since 1993 (McMillan, K., *et al.*, 1993). In their research, the substrate L-arginine and its analogues were shown to interact with the heme group in a specific dose and concentration-dependent manner. The following tests in this study were carried out on a recording spectrophotometer. The compounds used in binding assays were imidazole, L-arginine, D-arginine, L-lysine, the NO synthetic intermediate NOHA, NOS inhibitors NA, S-EITU, LNMMA, 1400W and L-NIL. All SANOS kinetic experiments were carried out multiple times with different batches of enzyme in triplicate using murine iNOS as a control. The results are shown in **Figure 5.1**, **5.2**, **5.3**, **5.4**, **5.5**, **5.6**, **5.7**, **5.8**, **5.9**, and **5.10**. The Ki values are summarised in **Table 5.1**. The profile of SANOS binding to L-arg, NOHA, NA, and LNMMA, shows more similarity to iNOS rather than nNOS. However, there are differences in binding to imidazole, S-EITU, and L-NIL comparing SANOS with the other NOS-domains used in this study. SANOS possesses a higher Ki value than iNOS and nNOS. For imidazole binding, the imidazole shift for SANOS at 396 nm is not as great as that for iNOS. Both SANOS and iNOS have similar Ki's for arginine (15.6

μM and 19.3 μM respectively), and this is higher than nNOS (2.9 μM). NOHA is an intermediate of NO synthesis generated by the NOS isozymes, and SANOS binds to NOHA with a Ki of 3.2 μM, intermediate between iNOS (4.7 μM) and nNOS (1.7 μM).

Various NOS inhibitors have been studied over the past few years, such as NA (Furfine, E.S., *et al.*, 1993; Rondouin, G., *et al.*, 1993; Klatt, P., *et al.*, 1994); S-EITU (Garvey, E.P., *et al.*, 1994; Nakane, M., *et al.*, 1995); L-NMMA (Stenger, S., *et al.*, 1995; Fukatsu, K., *et al.*, 1996); 1400W (Garvey, E.P., *et al.*, 1997; Thomasen, L.L., *et al.*, 1997; Hamilton, L.C., *et al.*, 1998); and L-NIL (Southan, G.J., *et al.*, 1996; Wolff, D.J., *et al.*, 1998; Grant, S.K., *et al.*, 1998; Handy, R.L.C., *et al.*, 1998). Among them NA is regarded as an nNOS specific inhibitor. In this study, the heme domain of rat nNOS has a Ki of 0.81 μM for NA binding, which is much lower than iNOS (4.9 μM) and SANOS (7.5 μM). A comparison of L-NMMA binding to SANOS, iNOS, and nNOS shows quite a range of binding affinities, with Ki values ranging from 9.1 μM, to 5.4 μM and to 0.48 μM respectively.

S-EITU has been reported to be a potent competitive inhibitor of human NOS isozymes, with Ki values of 17 nM, 36 nM, and 29 nM for the iNOS, eNOS and nNOS enzymes respectively (Garvey, E.P., *et al.*, 1994). In this study murine iNOS and rat nNOS gave Ki values of 0.39 μM and 0.13 μM respectively for S-EITU binding. Interestingly, the value for SANOS was significantly higher at 5.7 μM.

In summary, SANOS was very similar with respect to the binding of most of the inhibitors and substrates tested.

In 1997, Ghosh, D.K., *et al.* determined that amino acid residues 66 to 114 of iNOS were involved in subunit dimerisation and BH_4 interaction. Importantly they demonstrated that the iNOS heme domain had enzymatic activity, and could convert NOHA to NO and citrulline in the presence of SOD and H_2O_2. The ability of SANOS to carry out the same reaction was tested using the murine iNOS heme domain as a

control. **Table 5.2** and **Figure 5.11, 5.12** show the results of hydrogen peroxide oxidation of NOHA by these two proteins.

For the iNOS heme domain, L-NMMA inhibited around 50% of the reaction, (from 110 μM/μg protein to about 40 μM/μg). NA did not show any significant effect on the NO production by the iNOS heme domain. NO could not be measured following treatment with S-EITU or without the addition of NOHA. The absence of BH_4 causes a great reduction in the synthesis of NO by the iNOS domain.

SANOS produces a different profile compared with iNOS in the H_2O_2 oxidation test. Overall there are four differences:

(i) the NO generation was much less with SANOS than with the iNOS heme domain;

(ii) the NO production was dependent on the amount of protein at the initial point. There is no increased difference if reactions are allowed to proceed longer than 30 minutes.

(iii) the three tested NOS inhibitors, L-NMMA, S-EITU, and NA reduced the NO production to 50% of the level compared with reactions in the absence of inhibitor;

(iv) the NO generated by SANOS was not affected by the presence of BH_4 in the reaction mix.

7.5. General discussion

7.5.1. Data base searching

In 1990, Altschul, *et al.* published their study comparing the sequences of DNA and protein, giving rise to 'basic local alignment search tool' (BLAST). BLAST can be implemented in a number of ways and applied in a variety of contexts including straightforward DNA and protein sequence database searches, motif searches, gene identification searches, and in the analysis of multiple region of similarity in long DNA sequences. They had implemented a shared memory version of BLAST that

loads the compressed DNA file into memory once, allowing subsequent searches to skip this step, and they also suggested a similar algorithm for comparing a DNA sequence to the protein database, allowing translation in all six reading frames (Altschul, S.F., *et al.*, 1990).

The BLAST approach permits the construction of extremely fast programs for database searching that have the further advantage of amenability to mathematical analysis. Variations of the basic idea as well as alternative implementations, such as those described above, can adapt the method for different contexts. Given the increasing size of sequence databases, BLAST can be a valuable tool for the molecular biologist (Altschul, S.F., *et al.*, 1990).

Searching for NOS-like protein(s) from finished and unfinished microbial genome projects suggested that a NOS heme domain-like protein family may exist in Gram-positive bacteria (**Figure 4.4**). From Figure 4.4, this family of proteins seems to include the heme domain of NOS, but omit the BH_4 binding metal centre.

While this thesis was being revised, the whole genomic DNA sequence of *S.aureus* N315 was published by Kuroda, M., *et al.*, 2001. A protein with extensive homology to NOS was initially found by database searching in the unfinished genome sequence, and this sequence is present in the finished sequence. Through out this work, the protein has been called SANOS because of the similarity to mammalian NOS isoforms. In addition, the protein referred to as STAPHRED in this thesis (because of sequence similarity to the C-terminal reductase domain of NOS) is annotated as having similarity to a sulfite reductase flavoprotein in the finished *S.aureus* genomic DNA sequence.

The NOS heme domain is important catalytically as it can bind to heme and the NO synthesis substrate L-arginine. The amino acid residue C197 in human iNOS is necessary to bind heme. When candidate bacterial NOS-like sequences were aligned with human iNOS, (especially around the C197 residue in human iNOS), the peptide sequences, shown below, are seen to be highly conserved.

```
HiNOS       191 WRNAPRCIGRIQW
anthra       29 WRNSNRCIGRLFW
Bacha        60 WRNSNRCIGRLFW
Radio        57 WRNSTRCVGRLYW
SANOS        56 WRNSNRCIGRLFW
yflM         33 WRNSNRCIGRLFW
                ***: **:**: *
```

According to the data generated by Chen P *et al.* in 1997, Glu 374 in human iNOS was confirmed as the arginine-binding residue. An alignment of the arginine-binding region between human iNOS and bacterial NOS is listed below. The data shows the conservation in peptide sequences shared among the NOS-like proteins.

```
HiNOS       366 FNGWYMGTEIGVRDL
Anthra      202 FNGWYMGTEIGARNL
Bacha       234 FNGWYMGTEIGARNL
Radio       214 FSGWYVQTEIAARDL
SANOS       163 FNGWYMVTEIGVRNF
YFLM        208 FNGWYMGTEIGARNL
                *.***: ***..*::
```

From the above alignments, the NOS-like proteins in bacteria may have heme and arginine binding properties.

In their research of NOS subunit dimerisation, Ghosh (1997) determined that amino acids 66 to 114 in iNOS are responsible for NOS dimerisation and BH_4 binding. The sequence of the bacterial NOS-like protein starts after residue 114 in iNOS (**Figure 4.4**) strongly suggesting that the bacterial NOS-like protein may not be able to bind BH_4. However, the crystal structure of SANOS demonstrates that the protein can exist as a dimer and could bind to a co-factor other than BH_4. In fact, the site occupied by BH_4 in iNOS is occupied by NAD in the bacterial structure.

The genomic sequence of *B. subtilis* was finished in 1997 (Kunst, F., *et al.*, 1997), and contains a protein-coding sequence (named *yflM)* that is designated as similar to nitric oxide synthase. The BLAST and the alignment of *yflM* with other NOS-like proteins is shown in **Figures 3.1, 3.2, 3.3** and **4.4**. This analysis highlights similar features of the *yflM*–encoded protein and SANOS from *S.aureus*, along with other bacterial NOS-like sequences.

A protein with identity to the C-terminal reductase domain of NOS was also recognised in the unfinished *S.aureus* genome, and was called STAPHRED (**Appendix 3**). When NCPR and the reductase domain of NOS, were aligned with STAPHRED, the high degree of similarity suggests that STAPHRED may be an NCPR-like protein.

Overall, the data suggests that two separate domains of NOS can be identified in the *S.aureus* genome.

7.5.2. The properties of bacterial NOS-like proteins

7.5.2.1. YFLM

When the anti-*yflM* anti-serum was used to recognise the expressed *yflM* protein in *E. coli* and *B. subtilis*, two bands cross-reacted with the anti serum. One of them was around 40 kDa, the other was around 27 kDa. Denatured purified *yflM*-encoded protein shows a single band around 40 kDa.

In Northern blots, *yflM* RNA was found to be of a similar size in both *E. coli* and *B. subtilis* (**Figure 3.10**). This suggested that *yflM* RNA was expressed, and taken together with the antibody data, supports the hypothesis that a *yflM*-encoded protein is made in *B. subtilis*. It is still unclear whether or not the *yflM*–encoded protein undergoes cleavage *in vivo*, or if this finding is an artefact of the cell extraction procedure.

In 1996, Liu, Q., *et al.* described that an nNOS (558-721) fragment was expressed in *E. coli* and found to bind NA, albeit with lower affinity than the intact oxygenase domain (nNOS 220-721). However, BH_4-dependence of NA binding to nNOS 558-721 fragment was not observed. In contrast, nNOS 220-558 was found to be incapable of binding either BH_4 or NA. The authors interpreted these finding to suggest that the nNOS (558-721) fragment contains sufficient sequence for arginine binding, but that an extension N-terminally of up to 338 amino acids is needed for

high-affinity binding and regulation by BH_4 (Liu, Q., *et al.*, 1996). Amino acid 125 in the *yflM*-encoded protein is located before amino acid 558 in nNOS (**Figure 1.2**), suggesting that the smaller *yflM*–encoded protein may not be able to bind L-arginine. Interestingly, in chapter 5, a NOS-like protein (SANOS) is described that has functional similarity with mammalian NOS heme domains. The *yflM*–encoded protein shares more than 55% identity with SANOS, and is very similar in length. Given this finding, it is possible that the *yflM*-encoded protein possesses similar properties to SANOS. However, the role of the *yflM*-encoded protein in *B. subtilis* is still a mystery.

7.5.2.2. SANOS

DNA sequencing showed that the sequence of the gene encoding SANOS identified in this study was identical with the sequence reported in the data-base (**Figure 4.6**). Recombinant protein expression was carried out using the vector pCWori, and expression studies were carried out by Western and Northern blotting.

Northern blot showed that the gene encoding SANOS was expressed in *S.aureus*. Interestingly when making recombinant protein, the OD_{600} of the 48 hours induced-culture of SANOS in *E. coli* reached an OD600 of around 23. The expression level of SANOS however was not as high as murine iNOS in *E. coli*. To purify recombinant SANOS, the protocol used for the murine iNOS heme domain was modified in accordance with Rodriguez-Crespo, I., *et al.* (1996). The recovery of SANOS was 25 mg/litre of induced culture (compared to 60 mg/litre for the murine iNOS heme domain, Lowe PN, data not shown). Purified SANOS appears as a dark brown solution, similar to the murine iNOS heme domain.

As part of a collaborative study, purified SANOS was sent to Dr David Stammers (Oxford University) for crystallisation studies (see appendix). Structural studies show that SANOS is a dimeric heme protein and the BH_4 found in mammalian NOS structures is replaced by NAD.

7.5.2.3. STAPHRED

NOS proteins comprise two domains, and these have been characterised following trypsin digestion (Lowe, P.N., *et al.*, 1996). Importantly, these two domains can function separately (Pufahl, R.A., *et al.*, 1995; Ghosh, D.K., *et al.*, 1997; Stuehr, D.J., 1997). The two domains are linked by an oligopeptide motif that is capable of calcium/calmodulin binding (Lowe, P.N., *et al.*, 1996). The N-terminal half of the protein has been characterised as a unique heme domain that is not found in any other protein (reviewed in Titheradge, M.A., *et al.*, 1998). The C-terminal domain is an NADPH cytochrome P450 reductase like protein, which binds FMN, FAD and needs NADPH functionally. This reductase domain of NOS functions as an electron donor in the enzymatic generation of NO by NOS. The electrons are transferred from NADPH to FAD, FMN and to the electron acceptor heme group (in the N-terminal domain), which oxidises L-arg to NO and L-citrulline (Stuehr, D.J., *et al.*, 1997). The two domains of NOS (N-terminal heme domain, and C-terminal reductase domain) have been purified separately, and used in reconstitution experiments to attempt to generate NOS activity. Importantly, these experiments demonstrate that NOS activity can be successfully regenerated when domains are mixed (Ghosh, D.K., *et al.*, 1995; Chen, P.F., *et al.*, 1996). These findings support the hypothesis that, while functional NOS activity can be generated by the full-length enzyme, it may also be possible to generate NOS activity with functional domains and these domains may arise from the expression of separate genes.

The NOS C-terminal reductase domain is a cytochrome P450 reductase-like protein, Wang M *et al.*, 1997. The molecule is composed of four structural domains: (from the N- to C-termini) the FMN-binding domain, the connecting domain, and the FAD- and NADPH-binding domains. The FMN-binding domain is similar to the structure of flavodoxin, whereas the two C-terminal dinucleotide-binding domains are similar to those of ferredoxin-$NADP^+$ reductase (FNR). The connecting domain, situated between the FMN-binding and FNR-like domains, is responsible for the relative orientation of the other domains, ensuring the proper alignment of the two flavins necessary for efficient electron transfer (Wang, M., *et al.*, 1997). The biochemistry of

the NOS reductase domain has been summarised recently in a review by Stuehr (1997). The flavin and NADPH consensus binding regions are all located within the carboxyl-half of NOS, making this region of NOS structurally homologous with the dual-flavin enzymes NADPH-cytochrome P450 reductase, the falvoprotein subunit of sulfite reductase, and the C-terminal reductase domain of the bacterial cytochrome P-450 $_{BM-3}$. Because these proteins all function to transfer electrons derived from NADPH to an endogenous heme group or associated hemeprotein, the C-terminal half of NOS was proposed to function as a reductase domain that transfers electrons from NADPH to another redox component located in a different region of the protein. This redox component was subsequently identified to be iron protoporphyrin IX (heme), which resides within the N-terminal oxygenase domain of NOS.

The main reason to express the STAPHRED protein was to test the hypothesis that *S.aureus* was capable of generating NOS by expression of two separate proteins (an N-terminal heme domain, and a C-terminal reductase domain) that correspond to the full-length NOS isoform. Ghosh, D.K., *et al.* in 1995 and Chen, P.F., *et al.* in 1996 had reported the reconstitution of NOS activity by mixing separated domains of murine iNOS and bovine eNOS. These results support the hypothesis that NO synthesis could be conducted by separate NOS domains. Additional studies showed that most of the NADPH oxidation that occurred in the mixed-domain system was uncoupled to heme iron reduction. This suggests that the separated NOS reductase and oxygenase domains can only interact sub-opimally in solution to catalyse NO synthesis. Electron transfer between the separate domains is inefficient and probably represents a limiting factor for NO synthesis, which requires a sequential transfer of three electrons between the reductase and oxygenase domains to convert L-arg to L-citrulline and NO (Stuehr, D.J., 1997).

The sequence of the gene encoding STAPHRED was identical with that reported in the database. When it was expressed in *E. coli* (using pET28a), the expression level was low, and it was difficult to identify a band by Coomassie blue staining in SDS-PAGE (**Figure 6.6**). The attempt to purify STAPHRED was carried out using an

FPLC system, but the recovery was too small (**Figure 6.7**) to proceed for further functional tests.

To try to reconstitute NOS activity from the two bacterial NOS domains, co-expression experiment was performed using recombinant plasmids in different vectors, (SANOS in pCWori and STAPHRED in pACYC184). These plasmids have different origins of replication, and can be propagated in the same bacterial host. The co-expression results are shown in **Figure 6.8**. Only SANOS could be detected following the co-expression, STAPHRED was not even detectable using an anti-histidine antibody.

It is always difficult to determine why any particular protein is not well expressed in *E. coli*. It is possible for example that codon usage is not optimal for STAPHRED to be expressed, or that STAPHRED expression in *E. coli* is toxic. Interestingly, Choi, W.S., *et al.* (1997) reported the measurement of NOS activity in lysates of *S.aureus*, although no NOS gene was characterised. The work reported in this thesis demonstrates that two proteins corresponding to the N- and C-terminal domains of NOS can be found in *S.aureus*. These proteins may account for this measurable NOS activity. Ultimately, the best way to test this theory would be to attempt to reconstitute NOS activity by mixing the two separate NOS-like proteins from *S.aureus*. However, the expression and yield of STAPHRED made these reconstitution experiments with the heme domain difficult to carry out. An attempt was made to co-express the two NOS-like proteins in the same *E. coli* background. To carry out this experiment, STAPHRED was expressed under the control of the T_7 promoter in the vector pACYC184. The two recombinant plasmids expressing SANOS (pCWori) and STAPHRED (pACYC184) were transformed into BL21 (DE3) for co-expression. The colonies with both ampicillin and tetracycline resistance were selected. Unfortunately, using this co-expression system SANOS was expressed at comparatively low levels, while STAPHRED could not be detected, even when using anti-histidine antibody (**Figure 6.8**). The detailed mechanism(s) of co-expression need to be further studied.

7.5.3. Characterisation of SANOS

Gorren, A.C.F., *et al.* (1996) reported that nNOS-containing heme, but not BH_4, had been shown to exist in a dimeric form, but could not catalyse citrulline formation. The absorption spectrum of the BH_4-free enzyme is quite different to that of the native enzyme (which normally, contains about 1 mol of BH_4 per mole of dimer), with a maximum at 418 nm instead of 398 nm. By analogy with cytochrome P450, this difference reflects a shift in the spin-state of the heme iron, which is low-spin in the BH_4-free enzyme but predominantly high-spin in the native enzyme.

When the SANOS heme status was measured, the imidazole-shift paralleled the murine iNOS heme domain. The maximum absorption spectrum was shifted from 422 nm to 398 nm in the presence of BH_4. This shifting suggested that the SANOS heme retained the same status as the iNOS and nNOS heme domains.

To test L-arginine binding, and BH_4 influences, the spectrums of SANOS shifted by L-arginine with and without BH_4 were recorded (**Figure 5.3**). Interestingly, the SANOS spectrum maximum absorption was shifted by L-arginine from 418nm to 398 nm, and BH_4 did not affect the shifting.

Presta *et al.,* (1998) carried out a comparative study of the effect of different pterin groups on supporting electron transfer, catalysis, and subunit dimerisation of iNOS. During NO synthesis, all pterin-dependent steps up to, and including, heme iron reduction can take place independent of the pterin ring oxidation state, indicating that the requirement for fully-reduced pterin occurs at a point in catalysis beyond heme iron reduction (Presta, A., *et al.*, 1998).

Combining trypsin digestion and deletion-mutation analysis, residues 66 to 144 of the iNOS heme domain have been shown to be involved in BH_4 interaction and subunit dimerisation. BH_4 binding appears to stabilise the protein structure in this region, and through doing so activates iNOS for NO synthesis (Ghosh, D.K., *et al.*, 1997).

From the alignment of SANOS with human iNOS, as shown in **Figure 4.1**, SANOS starts from amino acid 130, corresponding to the peptide sequence of iNOS. In human iNOS, amino acids 107 to 112 exhibits the sequence motif CKSKLC. This motif has been verified (by X-ray structural studies on eNOS) to co-ordinate BH_4-binding (Raman, C.S., *et al.*, 1998). As this sequence is absent from SANOS, this alignment suggests that SANOS may not be able to bind BH_4.

Cho, H.J., *et al.*, (1995), have used site-directed mutagenesis to show that Gly-450 and Ala-453 in the heme domain of murine iNOS were critical for NO production, dimer formation, and BH_4 binding. **Appendix 5** shows that these residues are common in virtually all of the NOS protein sequences in GeneBank, with the exception of the SANOS and Lyms (pond snail) sequences.

Alderton, WK (1998) studied the binding kinetics of the heme domain of rat nNOS, and many characteristics of the arginine-binding site of NOS are conserved in the heme-binding domain. Additionally, apparent Kd values were compared and were found to be similar for the inhibitors, L-N^G-monomethylarginine, S-ethylisothiourea, N-iminoethyl-L-ornithine, imidazole, 7-nitroindazole and 1400W (N-[3-(aminomethyl) -benzyl]acetamidine).

After SANOS was identified as a heme protein, it was important to study the binding of substrates and inhibitors, and to assess enzyme activity. Optical difference spectrophotometry was used to measure substrate binding to SANOS, and difference-spectra have been used to assess the heme status of proteins for many years (Schenkman, J.B., *et al.* 1967). McMillan *et al.* (1993) demonstrated that optical difference spectrophotometry could be used as a probe of rat brain NOS heme-substrate interactions. In their research, the substrate L-arginine and the reaction intermediate NOHA were demonstrated to exhibit a specific and titratable interaction with the heme prosthetic group. These studies implicated the heme group as the reaction centre for the initial binding of substrate and the subsequent incorporation of oxygen to form both products, NO and citrulline. Interestingly, NOS exhibits type II difference spectra upon titration with imidazole, characterised by the appearance of a

peak at ~430 nm and a trough at ~395 nm, with a spectral binding constant of ~160 μM.

The appearance of difference spectra due to heme perturbation by the putative substrates or inhibitors constitutes direct evidence that the heme prosthetic group of rat brain NOS binds initially with the substrate, presumably prior to serving as the mono-oxygenase reaction centre in these enzyme (McMillan, K., *et al.*, 1993). In their work, the substrate L-arginine, the intermediate N^G-hydroxy-L-arginine, and the inhibitor N^G-methyl-L-arginine were all shown to interact with the heme prosthetic group in a specific and titratable manner, indicating that the substrate binds in the vicinity of the heme prior to electron transfer. Titration of NOS with L-arginine and NOHA in the presence of calcium/CaM showed no effect on the properties of the observed spectral perturbation or the respective binding constants (McMillan, K., *et al.*, 1993). These data suggest that the CaM binding domain is unlikely to be important in the substrate binding for NOS activity.

Apart from heme determination tests, imidazole has been used to measure heme status in NOS isoforms. The antifungal imidazole and its analogos were recognised as the first NOS inhibitor. Wolff *et al.* (1993) reported that imidazole increases the concentration of CaM required to activate the enzyme half-maximally and reduce the maximal velocity of citrulline formation. This inhibition was not reversed by increased concentrations of either the arginine substrate or (6R)-5,6,7,8-tatrahydro-L-biopterin. Later, the same group (Wolff, D.J., *et al.*, 1993, 1994a, 1994b) and Mayer, B. (1994) carried out further studies to identify the role of imidazole in NOS activation. The NOS used in these studies were the bovine brain CaM-dependent NOS (Wolff, D.J., *et al.*, 1993), the murine iNOS (Wolff, D.J., *et al.*, 1994), the constitutive bovine eNOS (Wolff, D.J., *et al.*, 1994), and porcine brain NOS (Mayer, B., *et al.*, 1994). In bovine brain NOS, imidazole inhibits the maximal velocity of citrulline formation by the enzyme, but does not alter the concentration of arginine, CaM, or (6R)-5,6,7,8-tetrahydro-L-biopterin required for expression of half maximal activity.

Imidazole had no effect on CaM-dependent reduction of cytochrome c by the enzyme at concentrations up to 50-fold higher than that inhibited citrulline formation. Imidazole inhibited CaM-dependent NADPH consumption by the enzyme with dissolved oxygen as the sole electron acceptor. So actually imidazole inhibits citrulline formation and oxygen reduction by acting as a sixth coordination ligand of the heme iron (Wolff, D.J., *et al.*, 1993). According to Mayer, M., (1994), imidazole acts as a heme-site inhibitor of NOS.

The addition of imidazole to NOS results in titration of the predominantly high-spin state species to the low-spin state. Approximately 75% conversion of the total enzyme present to the low-spin-state species was obtained in the presence of 1 mM imidazole. This conversion is likely to occur by formation of a hexa-coordinate heme iron, in which imidazole serves as the distal sixth ligand, with a concomitant displacement of bound substrate from the active site (McMillan, K., *et al.*, 1993).

Enzyme kinetic experiments showed that imidazole enhanced the apparent km for L-arginine without affecting maximal enzyme activity, and binding studies revealed that the inhibitor displaced the radioligand N^G-Nitro-L-[^{3}H]arginine in a concentration-dependent fashion. These results show that imidazole exerts its effects on NOS in an L-arginine competitive manner, and that the substrate site of the enzyme may be identical with the prosthetic heme group. The small molecule binds in a narrow cleft within the larger active-site cavity containing heme and BH_4. SEITU and L-arginine are hydrogen-bonded to a conserved glutamate (eNOS E361, iNOS E377). In fact experiments have shown that the active-site residues of iNOS and eNOS are nearly identical (Fischmann, T.O., *et al.*, 1999).

N^{ω}-hydroxy-L-arginine (NOHA) has been recognised as an intermediate of NO generation (Stuehr, D.J., *et al.*, 1991; Korth, H., *et al.*, 1994; Campos, K.L., *et al.*, 1995; Clague, M.J., *et al.*, 1997; Abu-Soud, H.M., *et al.*, 1997; Witteveen, C.F.B., *et al.*, 1998; Moali, C., *et al.*, 1998). Its production depends on the NADPH supported hydroxylation of L-arginine. NOS isoforms can use NOHA to generate NO and citrulline in the presence of other cofactors. Using H_2O_2 in place of O_2 and NADPH,

NOS enzymes have been shown to be capable of synthesising NO by using NOHA as substrate (Pufahl, R.A., *et al.*, 1995). Further research was performed by Ghosh, D.K., *et al.* in 1997, who used the heme domain of murine iNOS (residues 1-498) in the hydrogen peroxide supported oxidation reaction to generate NO from NOHA. Their work supported the hypothesis that the NOS heme domain is the catalytic centre for NO formation.

In this research, SANOS was tested for its ability to catalyse the formation of NO using NOHA as a substrate in the presence of SOD and H_2O_2. As shown in **Figure 5.11** and **Table 5.2**, SANOS generates around one sixth of the activity compared to the murine iNOS heme domain in converting NOHA to NO. There is no measurable activity when NOHA is absent from the reactions of both SANOS and murine iNOS. The catalytic activity and co-factor requirement of SANOS was different from murine iNOS, especially with regard to the BH_4 influence. BH_4 plays a crucial part in NO formation and dimer stabilisation in mammalian NOS isoforms (Stuehr, D.J. 1997). For murine iNOS, the conversion of NOHA to NO was inhibited completely when BH_4 was absent. Interestingly, for SANOS, BH_4 showed the contrary effect. Over a time-course, the rate of conversion of NOHA to NO by SANOS gradually increased without BH_4. NOS inhibitors (included as controls) were able to reduce the rate of NO-generation in SANOS and the mammalian NOS-domains. The P450/NADPH-cytochrome P450 reductase BM-3 from *B.megaterium* is a well-characterised heme protein, and was included as a negative control in this series of experiments. This protein was unable to show any NO formation from NOHA (data was not shown; Li, H., *et al.*, 1991; Gonvindaraj, S., *et al.*, 1994; Black, S.D., *et al.*, 1994) supporting the hypothesis that the reaction is confined to NOS-like proteins.

After SANOS was biochemically characterised, a recombinant plasmid was constructed for the generation of a SANOS mutant in *S.aureus* by allelic replacement. In recent years, the knock-out method has been used to study *S.aureus* survival and metabolism (Novick, R.P., *et al.*, 1993; Wu, S., *et al.*, 1996; Stranden, A.M., *et al.*, 1997; Bellido, J.L.M., *et al.*, 1997; Mei, J., *et al.*, 1997; Chan, P., *et al.*, 1998; Schwan, W.R., *et al.*, 1998; Ling, B., *et al.*, 1998; Perl, T.M., *et al.*, 1998; Archer,

G.L., 1998; Noble, W.C., 1998; Sulavik, M.C., *et al.*, 1998; Miyazaki, E., *et al.*, 1998; Foster, T.J., *et al.*, 1998; Clements, M.O., *et al.*, 1998; Clements, M.O., *et al.*, 1999; Kaatz, G.W., *et al.*, 1999; Nicholas, R.O., *et al.*, 1999; Su, C., *et al.*, 2000). Because NO is a fundamental molecule in many life processes, it will be interesting to determine if SANOS is playing an important role in *S.aureus* biology.

In conclusion, functionally SANOS can behave like a mammalian NOS heme-domain, and catalytically can generate NO from the NO intermediate NOHA in the presence of SOD and H_2O_2.

7.6. Limitations of the research

This is the first report of the characterisation of a bacterial NOS-like protein at the molecular level. While NOS-like enzymology has been described, the physiological importance of the protein has yet to be determined, however, this will have to be the subject of further studies.

7.6.1. YFLM

The solubility of the *yflM*–encoded protein expressed in *E. coli* was the main problem with the study of NOS from *B. subtilis*. As a consequence of this problem, insufficient protein could be generated for any meaningful biochemical study.

7.6.2. SANOS and STAPHRED

While recombinant SANOS and STAPHRED could be expressed in *E. coli*, STAPHRED could only be produced at low levels. The expression of Gram-positive genes in Gram-negative bacteria has been reported by a number of researchers (for example, Trieu-Cuot, P., *et al.*, 1985; Brisson-Noel, A., *et al.*, 1988; Helmann, J.D., *et al.*, 1989; Tseng, H.C., *et al.*, 1990). However, all proteins are different, and it is impossible to determine beforehand how successful a protein-expression experiment

is likely to be. As an alternative strategy, (for future work) a Gram-positive expression system could be used to express STAPHRED.

7.7. Application

This research has characterised a novel member of the NOS family. Interestingly, similar binding kinetics and catalysis is observed between SANOS and mammalian NOS heme-domains. Importantly, these observations are supported by the analysis of the crystal structure of SANOS, which is very similar to that described for bovine eNOS and murine iNOS (Appendix 5 and 9). There are however important differences between the bacterial NOS and mammalian NOS heme domains, especially in the ability to bind CaM and BH_4.

7.8. Conclusion and future work

A family of NOS-like proteins was found in a variety of Gram-positive bacteria and one of them, SANOS from *S.aureus*, was studied in detail for this project. This is the first time that a bacterial NOS-like protein has been characterised at the molecular biological level. The bacterial NOS-like domain shows many similar features compared with mammalian NOS-domains. For example, like mammalian NOS heme domains, SANOS can generate NO from the NO synthesis intermediate NOHA in the presence of SOD and H_2O_2. Also in common with mammalian NOS heme domains, the crystal structure shows that SANOS is a dimer, however, NAD is found at the dimer interface instead of the usual BH_4. The availability of a crystal structure is very useful for structure-function studies employing site-directed mutagenesis, and these future experiments will shed further light on the enzymology of this interesting protein.

For other future experiments, knock-out constructs will be useful to attempt to study the role of SANOS in the biology of *S.aureus*. Initial attempts have already been made to generate an allelic replacement construct for SANOS, but the process of making mutants in *S.aureus* is not as straight forward as in *E. coli*.

Overall this project has set the ground-work for the further study of bacterial NOS-like domains.

REFERENCES

Abu-Soud, H.M., Feldman, P. L., Clark, P., *et al.* (1994). Electron transfer in the nitric oxide synthase: Characterisation of L-arg analogs that block heme iron reduction. *J. Biol. Chem.* **269**. 32318-26.

Abu-Soud, H. M., Loftus, M., and Stuehr, D. J. (1995). Subunit dissociation and unfolding of macrophage NO synthase: relationship between enzyme structure prosthetic group binding, and catalytic function. *Biochemistry* **34**. 11167-11175.

Abu-Soud, H. M., Presta, A., Mayer, B., *et al.* (1997). Analysis of neuronal NO synthase under single-turnover conditions: conversion of N-hydroxyarginine to nitric oxide and citrulline. *Biochemistry* **36**. 10811-10816.

Adachi, H., Iida, S., Oguchi, S. (1993). Molecular cloning of a cDNA encoding an inducible calmodulin-dependent nitric-oxide synthase from rat liver and its expression in COS 1 cells. *Eur. J. Biochem.* **217**. 37-43.

Adams, V., Krabbes, S., Jiang, H., *et al.* (1998). Complete coding sequence of inducible nitric oxide synthase from human heart and skeletal muscle of patients with chronic heart failure. Unpublished.
Homo sapiens heart muscle inducible nitric oxide synthase (iNOS) mRNA, Complete cds. Accession AF051164.

Adler, H., Adler, B., Peveri, P., *et al.* (1996). Differential regulation of inducible nitric oxide synthase production in bovis and caprine macrophages. *J. Infect. Dis.* **173**. 917-978.
Capra hircus inducible nitric oxide synthase mRNA, partial cds. Accession U29085.

Aguan, K., and Weiner, C. P. Unpublished.
Ovis aris nitric oxide synthase (nNOS) gene, partial cds. Accession U76739.

Aguan, K., and Weiner. CP. Unpublished.
Ovis aris nitric oxide synthase (eNOS) gene, partial cds. Accession U76738.

Akaike T, Maeda H. (2000) Nitric oxide and virus infection. *Immunol.* **101**:300-308

Alderton, W. A., Boyhan, A., Lowe, P. N. (1998). Nitroarginine and tetrahydro-biopterin binding to the heme domain of neuronal nitric oxide synthase using a scintillation proximity assay. *Biochem. J.* **332**. 195-201.

Altschul, S. F., Gish, W., Miller, W., *et al.* (1990). Basic Local Alignment Search Tool. *J. Biol. Chem.* **215**. 403-20.

Anken, R. H., Sorger, I., Bremen, D., *et al.* (1996). NADPH-diaphorase reactivity in the Mauthner cells of the swordtail fish, Xiphophorus helleri. *Neurosci. Lett.* **206**. 49-52.

Archer, G. L. (1998). *Staphylococcus aureus*: a well-armed pathogen. *Clinical Infec. Dis.* **26**. 1179-81.

Baek, K. J., Thiel, B. A., Lucas, S., *et al.* (1993). Macrophage nitric oxide synthase subunits. *J. Biol. Chem.* **28**. 21120-21129.

Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991). Expression and enzymatic activity of recombinant cytochrome P450 17α-hydroxylase in *Escherichia coli. Proc. Natl. Acad. Sci. U.S.A.* **88**. 5598-5601.

Barnes, P. J. (1995). Nitric oxide and airways disease. *Ann. Med.* **27**. 289-93.

Bellido, J. L. M., Manzanares, M. A. A., Guirao, G. Y., *et al.* (1997). In vitro activities of 13 fluroquinolones against *Staphylococcus aureus* isolates with characterized mutations in *gyrA*, *gyrB*, *gylA*, and *norA* and against wild-type isolates. *Antimicro. Agent. Chemother.* **43**. 966-968.

Beltran, B., Mathur, A., Duchen, M. R., *et al.* (2000). The effect of nitric oxide on cell respiration: a key to understanding its role in cell survival or death. *Proc. Natl. Acad. Sci. U.S.A.* **97**. 14602-07.

Berka, V., Chen, P. F., and Tsai, A. (1996). Spatial relationship between L-arginine and Heme binding sites of endothelial nitric oxide synthase. *J. Biol. Chem.* **271**. 33293-33300.

Black, S. D., Linger, M. H., Freck, L. C., *et al.* (1994). Affinity isolation and charac - terisation of cytochrome P450 102 (BM-3) from barbiturate-induced *Bacillus megaterium. Arch. Biochem. Biophys.* **310**. 126-33.

Black, S. M., Ortiz de Montellano. P. R. (1995). Characterisation of rat neuronal nitric oxide synthase expressed in *Saccharomyces cerevisiae. DNA Cell. Biol.* **14**. 789-94.

Boyhan, A., Smith, D., Charles, I. G., *et al.* (1997). Delineation of the arginine- and tetrahydrobioterin-binding sites of neuronal nitric oxide synthase. *Biochem. J.* **323**. 131-139.

Bredt, D. S., Hwang, P. M., Glatt, C. E., *et al.* (1991). Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature.* **351**. 714-718.

Bredt, D. S. and Snyder, S. H. (1994). Nitric oxide: a physiological messenger molecule. *Annu. Rev. Biochem.* **63**. 175-195.

Brenman, J.E., Christopherson, K.S., Craven, S.E., *et al.* (1996). Cloning and characterisation of postsynaptic density 93, a nitric oxide synthase interacting protein. *J. Neurosci.* **16**.7407-15.

Brenman, J.E., Chao, D.S., Gee, S.H., et al. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell.* **84**.757-67.

Brisson-Noel, A., Arthur, M., and Courvalin, P. (1988). Evidence for natural gene transfer from Gram-positive cocci to *Escherichia coli. J. Bacteriol.* **170**. 1739-45.

Brown, G. C. and Cooper, C. E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS. Lett.* **356**. 295-98.

Bruning, G., Katzbach, R. and Mayer, B. (1995). Histochemical and Immunocyto-chemical localisation of nitric oxide synthase in the central nervous system of the goldfish, *Carassius aurarus. J. Comp. Neurol.* **358**. 353-382.

Bruning, G. and Mayer, B. (1996). Localisation of nitric oxide synthase in the brain of the frog, *Xenopus laevis. Brain Res.* **741**. 331-343.

Busconi, L. and Michel, T. (1995). Recombinant endothelial nitric oxide synthase: post-translational modifications in a baculovirus expression system. *Mol. Pharmacol.* **47**. 655-9.

Calaycay, J. R., Kelly, T. M. and MacNaul, K. L. (1996). Expression and immuno-affinity purification of human inducible nitric-oxide synthase. *J. Biol. Chem.* **271**. 28212-28219.

Campos, K. L., Giovanelli, J. and Kaufman, S. (1995). Characteristics of the nitric oxide synthase-catalyzed conversion of arginine to N-hydroxyarginine, the first step in the enzymatic synthesis of nitric oxide. *J. Biol. Chem.* **270**. 1721-1728.

Chabin, R. M., McCauley, E., Calaycay, J. R., *et al.* (1996). Active-site structure analysis of recombinant human inducible nitric oxide synthase using imidazole. *Biochemistry* **35**. 9567-9575.

Charles, I. G., Palmer, R. M. J., Hickery, M. S., *et al.* (1993). Cloning, characterisation and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. *Proc.Natl.Acad. Sci.,U.S.A.* **90**. 11419-11423.

Charles, I.G., Chubb, A., Gill, R., *et al.* (1993). Cloning and expression of a rat neuronal nitric oxide synthase coding sequence in a baculovirus/insect cell system. *Biochem. Biophys. Res. Commun.* **196**. 1481-1489.

Chen, P.F., Tsai, A., and Wu, K.K. (1994). Cysteine 184 of endothelia nitric oxide synthase is involved in heme coordination and catalytic activity. *J. Biol. Chem.* **269**. 25062-25066.

Chen, P. F., Tsai, A., and Wu, K.K. (1995). Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity. *Biochem. Biophys. Res. Commun.* **215.** 1119-1129.

Chen, P.F., Tsai, A., Berka, V., *et al.* (1996). Endothelial nitric oxide synthase: Evidence for bidomain structure and successful reconstitution of catalytic activity from two separate domains generated by a baculovirus expression system. *J. Biol. Chem.* **271**. 14631-14635.

Chen, P. F., Tsai, A.L., Berka, V., *et al.* (1997). Mutation of Glu-361 in human endothelial nitric oxide synthase selectively abolishes L-arginine binding without perturbing the behaviour of heme and other redox centres. *J. Biol. Chem.* **272**. 6114-18.

Chen, P. F., Foster, S. J., Ingham, E., *et al.* (1998). The *Staphylococcus aureus* alternative sigma factor σ^B controls the environmental stress response but not starvation survival or pathogenicity in a mouse abscess model. *J. Bacteriol.* **180**. 6082-89.

Chen, Y. and Rosazza, Y. P. (1994). A bacterial nitric oxide synthase from a *Nocardia species. Biochem. Biophys. Res. Commun.* **203**. 1251-1258.

Chen, Y. and Prosszza, J. (1995). Purification and characterisation of nitric oxide synthase (NOSNoc) from a *Nocardia* species. *J. Bacteriol.* **177**. 5122-5128.

Cho, H. J., Mertin, E., Xie, Q., *et al.* (1995). Inducible nitric oxide synthase: identification of amino acid residues essential for dimerisation and binding of tetrahydrobiopterin. *Proc.Natl. Acad. Sci., U.S.A.* **92**. 11514-11518.

Choi, W. S., Chang, M. S., Han, J. W., *et al.* (1997). Identification of nitric oxide synthase in *Staphylococcus aureus. Biochem. Biphys. Res. Commun.* **237**. 554-558.

Choi, W. S., Seo, D. W., Chang, M. S., *et al.* (1998). Methylesters of L-Arginine and -Nitro-L-arginine induces nitric oxide synthase in *Staphylococcus aureus. Biochem. Biophys. Res. Commun.* **246**. 431-435.

Cioni, C., Greco, A., Pepe, A., *et al.* (1997). Nitric oxide synthase in the caudal neuro -secretory system of the teleost Oreochromis niloticus. *Neurosci. Lett.* **238.** 57-60.

Clague, M. J., Wishnok, J. S. and Marletta, M.A. (1997). Formation of N-cynoornithine from N-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: implications for mechanism. *Biochemistry.* **36.** 14465-14473.

Clark, R. B., Kinsberg, E.R. and Giles, W. R. (1994). Histochemical localisation of nitric oxide synthase in the bullfrog intracardiac ganglion. *Neurosci. Lett.* **182**. 255-258.

Cleeter, M. W., Cooper, J.M., Darley-Usmar, V. M., *et al.* (1994). Reversible inhibition of cytochrome c oxidation, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS. Lett.* **345**. 50-54.

Clementi, E., Brown, G. C., Feelisch, M., *et al.* (1998). Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-nitricsylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl. Acad. Sci., U.S.A.* **95**. 7631-36.

Clementi, E., Brown, G. C., Foxwell, N., *et al.* (1999). On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc. Natl. Acad. Sci., U.S.A.* **96**. 1559-62.

Clements, M. O. and Foster, S. J. (1998). Stress resistance in *Staphylococcus aureus. Tren. Microbiol.* **7**. 458-62.

Clements, M. O., Watson, S. P., Poole, R. K., *et al.* (1998). CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. *J. Bacteriol.* **181**. 501-07.

Conforti, E., Torti, C., Malacrida, A. R., *et al.* (1999). Mature and developing visual system of ceratitis capitata (Diptera, tephritidae): histochemical evidence of nitric oxide synthase in the wild type and the white eye mutant strains. *Brain Res.* **843**. 1-11.

Crane, B. R., Arvai, A. S., Gachhui, R., *et al.* (1997). The structure of nitric oxide synthase: oxygenase domain and inhibitor complexes. *Science.* **278**. 425-431.

Crane, B. R., Arvai, A.S., Ghosh, D. K., *et al.* (1998). Structure of nitric oxide synthase oxygenase dimer with pterin and substrate. *Science.* **279**. 2121-2125.

Cubberley, R. R., Alderton, W. K., Boyhan, A., *et al.* (1997). Cysterin-200 of human inducible nitric oxide synthase is essential for dimerisation of heme domains and for binding of heme, nitroarginine and tetrahydrobiopterin. *Biochem. J.* **323**. 141-146.

Derst, C., Preising-Mueller, R., Rajan, S., *et al.* (1999). Cloning and sequencing of guinea pig NO synthases. Unpublished.
Cavia porcellus clone 2 endothelial nitric oxide synthase (NOS3) mRNA, Complete cds. Accession AF146041.

Fishmann, T.O., Hruza, A., Niu, X., *et al.* (1999). Structural characterisation of nitric oxide synthase isoforms reveals striking active-site conservation. *Nature Struct. Biol.* **6**. 233-242.

Forstermann, U., Boissel, J. and Kleinert, H. (1998). Expression control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS II). *FASEB. J.* **12**. 773-790.

Fossetta, J. D., Niu, X. D., Lunn, C. A., Zavodny, P.J., *et al.* (1996). Expression of human inducible nitric oxide synthase in *Escherichia coli. FEBS. Letters.* **379**. 135-138.

Foster, T. J. and Hook, M. (1998). Surface protein adhesions of *Staphylococcus aureus. Tren. Microbiol.* **6**. 484-88.

Fujisawa, H., Ougura, T., Kurashima, Y., *et al.* (1994). Expression of two types of nitric oxide synthase mRNA in human neuroblastoma cell lines. *J. Neurochem.* **63**. 140-145.

Fujisawa, H. unpublished.
Human mRNA for neuronal nitric oxide synthase, complete cds. Accession D16408.

Fukatsu, K., Saito, H., Fukushim, R., *et al.* (1996). Effects of three inhibitors of nitric oxide synthase on host resistance to bacterial infection. *Inflamm. Res.* **45**. 109-112.

Furfine, E. S., Harmon, M. F., Paith, J. E., *et al.* (1993). Selective inhibition of constitutive nitric oxide synthase by L-N-nitroarginine. *Biochemistry.* **32**. 8512-8517.

Gachhui, R., Presta, A., Bentley, D. F., *et al.* (1996). Characterisation of the reductase domain of rat neuronal nitric oxide synthase generated in the methylotrophic yeast *Pichia pastoris. J. Biol. Chem.* **271**. 20594-20602.

Gachhui, R., *et al.* (1997). Mutagenesis of acidic residues in the oxygenase domain of inducible nitric-oxide synthase identifies a glutamate involved in arginine binding. *Biochemistry* **36**. 5097-5103.

Garvey, E. P., Oplinger, J. A., Tanoury, G. J., *et al.* (1994). Potent and selective inhibitor of human nitric oxide. *J. Biol. Chem.* **269**. 26669-26676.

Garvey, E. P., Oplinger, J. A., Furfine, E. S., *et al.* (1997). 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. *J. Biol. Chem.* **272.** 4959-4963.

Geller, D. A., Freeswick, P. D, Nguyen, D., *et al.* (1994). Differential induction of nitric oxide synthase in hepatocytes during endotoxemia and the acute-phase response. *Arch. Surg.* **129**. 165-171.

Gerber, N. C., Ortiz de Montellano, P. R. (1995). Neuronal nitric oxide synthase: expression in *Escherichia coli,* irreversible inhibition by phenyldiazene, and active site topology. *J. Biol. Chem.* **270**. 17791-17796.

Gerber, N. C., Nishida, C. R. and Ortiz de Montellano, P. (1997). Characterisation of human inducible nitric oxide synthase expressed in *Escherichia coli. Arch. Biochem. Biophys.* **343**. 249-253.

Gonvindaraj, S., Li, H. and Poulos, T. L. (1994). Flavin supported fatty acid oxidation by the heme domain of *Bacillus megaterium* cytochromeP450BM-3. *Biochem. Biophysi. Res. Commun.* **203**. 1745-49.

Ghosh, D. K. and Stuehr, D. (1995). Macrophage NO synthase: characterisation of isolated oxygenase and reductase domains reveals a head-to –head subunit interaction. *Biochemistry* **34**. 801-807.

Ghosh, D.K., Abu-Soud, H. M. and Stuehr, D. J. (1995). Reconstitution of the second step in NOS synthesis using the isolated oxygenase and reductase domains of macrophage NO synthase. *Biochemistry* **34**. 11316-11320.

Ghosh, D. K., Abu-Soud, H. M. and Stuehr, D. J. (1996). Domains of macrophage NO synthase have divergent roles in forming and stabilising the active dimeric enzyme. *Biochemistry* **35**. 1444-1449.

Ghosh, D.K., Wu, C., Pitters, E., *et al.* (1997). Characterisation of the inducible nitric oxide synthase oxygenase domain identifies a 49 amino acid segment required for subunit dimerisation and tetrahydrobiopterin interaction. *Biochemistry* **36**. 10609-10619.

Grabowski, P. S. (1996). Unpublished.
O.mykiss NOS2 mRNA for inducible nitric-oxide synthase, complete cds. Accession X97013.

Grant, S. K., Green, B.G., Stiffey-Wilusz, J., *et al.* (1998). Structural requirements for human inducible nitric oxide synthase substrates and substrates analogue inhibitors. *Biochemistry* **37**. 4174-4180.

Griffith, O. W. and Stuehr, D.J. (1995). Nitric oxide synthase: Properties and catalytic mechanism. *Annu. Rev. Physiol.* **57**. 707-736.

Hamilton, L. C. and Warner, T. D. (1998). Interactions between inducible isoforms of nitric oxide synthase and cyclo-oxygenase in vivo: investigations using the selective inhibitors, 1400W and celecoxib. *Bri. J. Pharmacol.* **125**. 335-340.

Handy, R. L. C. and Moore, P. K. (1998). A comparison of the effects of L-NAME, 7-NI and L-NIL on carrageenan-induced hindpaw oedema and NOS activity. *Bri. J. Pharmacol.* **123**. 1119-1126.

Harwood, C.R. (1992). Bacillus subtilis and its relatives: molecular biological and industrial workhorses. *Trends. Biotechnol.* **10**.247-56.

Haverkamp, S. and Eldred, W. D. (1998). Localisation of nNOS in photoreceptor, bipolar and horizontal cells in turtle and rat retinas. *Neurorep.* **13**. 2231-2235.

Helmann, J. D., Wang, Y., Mahler, I., *et al.* (1989). Homologous metalloregulatory protein from both Gram-positive and Gram-negative bacteria control transcription of mercury resistance operons. *J. Bacteriol.* **171**. 222-29.

Hendriks, W. (1995). Nitric oxide synthase contains a discs-large homologous region (DHR) sequence motif. *Biochem. J.* **305**. 687-88.

Hibbs, J.B., Jr, Vavrin, Z., Taintor, R.R. (1987). L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol.***138**.550-65.

Hurst, W. J., Moroz, L. L., Gillette, M.U., *et al.* (1999). Nitric oxide synthase immunolabeling in the molluscan CNS and peripheral tissues. *Biochem. Biophys. Res. Commun.* **262**. 545-548.

Hylland, P., Nilson, G. E. (1995). Evidence that acetylcholine mediates increased cerebral blood flow velocity in crucian carp through a nitric oxide-dependent mechanism. *J. Cereb. Blood Flow. Metab.* **15**. 529-534.

Janssens, S. P., Simouchi, A., Quertermous, T., *et al.* (1992). Cloning and expression of a cDNA encoding human endothelium-derived relating factor/nitric oxide synthase. *J. Biol. Chem.* **267.** 22694-98

Jeong, Y. and Jim, J. (1997). Unpublished.
Oryctolagus cuniculus nitric oxide synthase mRNA, Complete cds. Accession U91584.

Kaatz, G. W., Seo, S. M. and Foster, T. J. (1999). Introduction of a *norA* promoter region mutation into the chromosome of a fluoroquinolone-susceptible strain of *Staphylococcus aureus* using plasmid integration. *Antimicrob. Agent. Chemothe.* **43.** 2222-24.

Karila, P., Messenger, J. and Holmgren, S. (1997). Nitric oxide synthase- and neuro-peptide Y-containing subpopulations of sympathetic neurones in the Coeliac ganglion of the Atlantic cod, Gadus morhua, revealed by immunohistochemistry and retrograde tracing from the stomach. *J. Auton. Nerv. Syst.* **66.** 35-45.

Karlsen, A. E., Andersen, H. U., Vissing, H., *et al.* (1995). Cloning and expression of cytokine- inducible nitric oxide synthase cDNA from rat islets of Langerhans. *Diabet.* **44.** 753-8.

Keinanen, R., Vartiainen, N. and Koistinaho, J. K. (1999). Molecular cloning and characterisation of the rat inducible nitric oxide synthase (iNOS) gene. *Gene.* **234.** 297-305.

Khanolkar-Young, S., Snowdon, D. and Lockwood, D. N. (1998). Immunocyto-chemical localisation of inducible nitric oxide synthase and transforming growth factor-beta (TGF-beta) in leprosy lesions. *Clin. Exp. Immunol.* **113.** 438-442.

Kim, I. B., Lee, E. J., Kim, K.Y., *et al.* (1997). Immunocytochemical localisation of nitric oxide synthase in the mammalian retina. *Neurosci. Lett.* **267.** 193-196.

Kimura, T., Shouno, O. and Matsumoto, G. (1997). NADPH-diaphorase containing cells and fibers in the central nervous system of squid, Loligo bleekeri Keferstein. *Life Sci.* **61**. 2375-2381.

Klatt, P., Schmidt, K. and Mayer, B. (1992). Brain nitric oxide synthase is a hemeoprotein. *Biochem. J.* **288**. 15-17.

Klatt, P., Schmidt, K., Brunner, F., *et al.* (1994). Inhibitors of brain nitric oxide synthase: binding kinetics, metabolism, and enzyme inactivation. *J. Biol. Chem.* **269**. 1674-1680.

Klatt, P., Schmidt, M., Leopold, E., *et al.* (1994). The pterine binding site of brain nitric oxide synthase: tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain. *J. Biol. Chem.* **269**. 13861-13866.

Klatt, P., Schmidt, K., Werner, E. R. and Mayer, B. (1996). Determination of nitric oxide synthase cofactors: heme, FAD, FMN and Tetrahydrobiopterin. *Meth. Enzymol.* **268**. 358-365.

Klatt, P., Pfeiffer, S., List, B. M., *et al.* (1996). Characterisation of heme-deficient neuronal nitric-oxide synthase reveals a role for heme in subunit dimerisation and binding of the amino acid substrate and tetrahydrobiopterin. *J. Biol. Chem.* **271**. 7336-7342.

Knowles, R. G. and Moncada, S. (1992). Nitric oxide as a signal in blood vessels. *Trend. Biochem. Sci.* **17**. 399-402.

Korneev, S. A., Piper, M. R., Picot, J., *et al.* (1998). Molecular characterisation of NOS in a mollusk: expression in a giant modulatory neuron. *J. Neurobiol.* **35**. 65-76. Lymnaea stagnalis nitric oxide synthase (NOS) mRNA, Complete cds. Accession AF012531.

Korth, H. G., Sustmann, R., Thater, C., *et al.* (1994). On the mechanism of the nitric oxide synthase-catalysed conversion of N-hydroxy-L-arginine to citrulline and nitric oxide. *J. Biol. Chem.* **269**. 17776-17779.

Kunst, F., Ogasawara, N., Moszer, I., *et al.* (1997). The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature.* **390**. 249-256.

Kurenni, D. E., Thurlow, G. A., Turner, R. W., *et al.* (1995). Nitric oxide synthase in tiger salamander retina. *J. Comp. Neurol.* **361**. 525-536.

Kuroda,M., Ohta, T., Uchiyama, I., *et al.* (2001). Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *Lance.* **357**. 1225-1240.

Laing, K. J., Grabowski, P. S., Belosevic, M. and Secombes, C. J. (1996). A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. *Immunol. Cell. Biol.* **74**. 374-379.
C.auratus mRNA for inducible nitric oxide synthase, complete cds. Accession X97603.

Laubach, V. E., Garvey, E. P., Sherman, P. A., *et al.* (1996). High-level expression of human inducible nitric oxide synthase in Chinese hamster ovary cells and characterisation of the purified enzyme. *Biochem. Biophys. Res. Commun.* **218**. 802-807.

Leake, L. D. and Moroz, L. L. (1996). Putative nitric oxide synthase (NOS)-containing cells in the central nervous system of the leech, Hirudo medicinalis: NADPH-diaphorase histochemistry. *Brain Res.* **723**. 115-124.

Lechner, F., Schutte, A., Von Bodungen, U., *et al.* (1999). Inducible nitric oxide synthase is expressed in joints of goats in the late stage of infection with caprine arthritis encephalitis virus. *Clin. Exp. Immunol.* **117**. 70-75.

Ledbetter, A. P., McMillan, K., Roman, L. J., *et al.* (1999). Low-temperature stabilisation and spectroscopic characterisation of the dioxygen complex of the ferrous neuronal nitric oxide synthase oxygenase domain. *Biochem.* **38**. 8014-8021.

Lee, K. H., Baek, M. Y., Moon, K. Y., *et al.* (1994). Nitric oxide as a messenger molecule for myoblast fusion. *J. Biol. Chem.* **269**. 14371-14374.

Li, H., Darwish, K. and Poulos, T. L. (1991). Characterisation of recombinant Bacillus *megaterium* cytochrome P-450 and its two functional domains. *J. Biol. Chem.* **266**. 11909-11914.

Licad-Coles, E., He, K., Yin, H., *et al.* (1997). Cytochrome P450 2C11: *Escherichia coli* expression, purification, functional characterisation, and mechanism-based inactivation of the enzyme. *Arch. Biochem. Biophysi.* **338**. 35-42.

Lin, A. W., Chang, C. C. and McCormick, C. C. (1996). Molecular cloning and expression of an avian macrophage nitric oxide synthase cDNA and the analysis of the genomic 5'-flanking region. *J. Biol. Chem.* **271**. 11911-11919.
Chicken macrophage nitric oxide synthase mRNA, Complete cds. Accession U46504.

Ling, B. and Berger-Bachi, B. (1998). Increased overall antibiotic susceptibility in *Staphylococcus aureus femAB* null mutants. *Antimicrob. Agent. Chemother.* **42**. 936-938.

List, B. M., Klatt, P., Werner, E. R., *et al.* (1996). Overexpression of neuronal nitric oxide synthase in insect cells reveals requirement of heme for tetrahydrobiopterin binding. *Biochem. J.* **315**. 57-63.

Liu, H. P., Leong, S. K. and Tay, S. S. (1994). Localisation of NADPH-diaphorase positive neurons in the pancreas of the mouse, rat, chick, kittle and monkey. *J. Hirnforsch.* **35**. 501-510.

Liu, J. and Parkinson, J. S. (1989). Role of CheW protein in coupling membrane receptors to the intracellular signaling system of bacterial chemotaxis. *Proc. Natl. Acad. Sci., U.S.A.* **86**. 8703-07.

Liu, Q. and Gross, S.S. (1996). Binding sites of nitric oxide synthase. *Meth. Enzymol.* **268**. 311-314.

Lowe, P. N., Smith, D., Stammers, D. K., *et al.* (1996). Identification of the domain of neuronal nitric oxide synthase by limited proteolysis. *Biochem. J.* **314**. 55-62.

Lowenstein, C. J., Glatt, C. S., Bredt, D.S., *et al.* (1992). Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci., U.S.A.* **89**. 6711-6715.

Luckhart, S. and Rosenberg, R. (1999). Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. *Gene.* **232**. 25-34.

Lyons, C. R., Orloff, G. J. and Cunningham, J. M. (1992). Molecular cloning and functional expression of an inducible nitric oxide synthase from a murine macrophage cell line. *J. Biol. Chem.* **267**. 6370-6374.
Murine nitric oxide synthase (MAC-NOS) gene, complete cds. Accession M84373.

Marletta, M. A. (1993). Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268**. 12231-4.

Martasek, P., Liu, Q., Liu, J., *et al.* (1996). Characterisation of bovine endothelial nitric oxide synthase expressed in *E coli. Biochem. Biophys. Res. Comm.* **219**. 359-365.

Martasek, P., Miller, R. T., Roman, L. J., *et al.* (1999). Assay of isoforms of *Estherichia coli*-expressed nitric oxide synthase. *Meth. Enzymol.* **301**. 71-78.

Masters, B. S. S., MicMillan, K., Sheta, E. A., *et al.* (1996). Neuronal nitric oxide synthase, a modular enzyme formed by convergent evolution: structure studies of a cysteine thiolate-liganded heme protein that hydrolates L-arginine to produce NO as a cellular signal. *FASEB. J.* **10.** 552-558.

Matsumoto, T., Mitchell, J. A., Schmidt, H. H., *et al.* (1992). Nitric oxide synthase in ferret brain: localisation and characterisation. *Br. J. Pharmacol.* **107**. 849-852.

Mayer, B., Klatt, P., Werner, E. R., *et al.* (1994). Identification of imidazole as L-arginine-competitive inhibitor of porcine brain nitric oxide synthase. *FEBS. Lett.* **350**. 199-02.

Mayer, B., Wu, C., Gorren, A. C. G., *et al.* (1997). Tethahydrobiopterin binding to macrophage inducible nitric oxide synthase: heme spin shift and dimer stabilisation by the potent pterin antagonist 4-amino-tetrahydrobiopterin. *Biochemistry.* **36**. 8422-8427.

McMillan, K. and Masters, B. S. S. (1993). Optical difference spectrophotometry as a probe of rat nitric oxide synthase heme-substrate interaction. *Biochemistry.* **32**. 9875-80.

McMillan, K. and Masters, B. S. S. (1995). Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: Identification of the heme-binding proximal thiolate ligand as cysteine-415. *Biochemistry.* **34**. 3686-3693.

Mei, J., Nourbakhsh F., Ford, C. W., *et al.* (1997). Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol. Microbiol.* **26**. 399-407.

Meyer, P., Champion, C., Schlotzer-Schrehardt, U., *et al.* (1999). Localisation of nitric oxide synthase isoforms in porcine ocular tissues. *Curr. Eye Res.* **18**. 375-380.

Miyazaki, E., Chen, J., Ko, C., *et al.* (1998). The *Staphylococcus aureus rsbW (orf159)* gene encodes an anti-sigma factor of SigB. *J. Bacteriol.* **181**. 2846-51.

Miyahara, K., Kawamoto, K., Sase, K., *et al.* (1999). Cloning and structural characterisation of the human endothelial nitric-oxide-synthase gene. *Eur. J. Biochem.* **223**. 719-726.
Homo sapiens endothelial nitric oxide synthase gene, Complete cds. Accession D26607.

Moali, C., Boucher. J., Sari, M., *et al.* (1998). Substrate specificity of NO synthase: detailed comparison of L-arginine, homo-L-arginine, their N-hydroxy derivatives, and N-hydroxyn-L-arginine. *Biochemistry.* **37**. 10453-10460.

Mock, B. A., Krall, M. M., Byrd, L. G., *et al.* (1994). The inducible form of nitric oxide synthase (NOS2) isolated from murine macrophages maps near the nude mutation on mouse chromosome 11. *Eur. J. Immunogene.* **21.** 231-238.

Moncada, S., Palmer, R. M. J. and Higgs, A. E. (1991). Nitric oxide: Physiology, Pathophysiology, and Pharmacology. *Pharmacol. Rev.* **43**. 109-142.

Moncada, S. and Higgs, A. E. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**. 2002-12.

Moroz, L. L. and Gillette, R. (1996). NADPH-diaphorase localisation in the CNS and peripheral tissues of the predatory ser-slug *Pleurobranchaea californica. J. Comp. Neurol.* **367**. 607-622.

Moss, D. W., Wei, X.Q., Liew, F. Y., Moncada, S. and Charles, I. G. (1995). Enzymatic characterisation of recombinant murine inducible nitric oxide synthase. *Euro. J. Pharmacol.* **289**. 41-48.

Muller, U., Bicker, G. (1994). Calcium-activated release of nitric oxide and cellular distribution of nitric oxide synthesising neurones in the nervous system of the locust. *J. Neurosci.* **14**. 7421-7528.

Muller, U. (1997). The nitric oxide system in insects. *Prog. Neurobiol.* **51**. 363-381.

Murtaugh, M. P., Pampusch, M. S., Harsch, S., *et al.* (1997). Unpublished.
Sus scrofa inducible nitric oxide synthase mRNA, Partial cds. Accession U59390.

Nakane, M., Schmidt, H. H., Pollock, J. S., *et al.* (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS. Lett.* **316**. 175-80.

Nakane, M., Klinghofer, V., Kuk, J. E., *et al.* (1995). Novel potent and selective inhibitors of inducible nitric oxide synthase. *Mole. Pharmacol.* **47**. 831-834.

Nakane, M., Pollock, J. S., Klinghofer, V., *et al.* (1995). Functional expression of three isoforms of human nitric oxide synthase in baculovirus-infected insect cells. *Biochem. Biophys. Res. Commun.* **206**. 511-7.

Narhi, L.O., Fulco, A.J. (1986) Characterisation of a catalytically self-sufficient 119,000-dalton cytochrome P-450 monooxygenase induced by barbiturates in *Bacillus megaterium. J. Biol. Chem.* **261**.7160-9.

Nathan, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB. J.* **6**. 3051-64.

Nathan, C. and Xie, Q. W. (1994). Nitric oxide synthase: roles, tolls, and controls. *Cell.* **78**. 915-918.

Nelson, D.R., Kamataki, T., Waxman, D.J., *et al.* (1993) The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA. Cell. Biol.* **12**.1-51.

Nicholas, R. O., Li, T., McDevitt, D., *et al.* (1999). Isolation and characterisation of a *sigB* deletion mutant of *Staphylococcus aureus. Infect. Immun.* **67**. 3667-69.

Nighorn, A., Gibson, N. J., Rivers, D. M., *et al.* (1998). The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projection neurones in the antennal lobe of Manduca Sexta. *J. Neurosci.* **18**. 7244-7255.
Manduca sexta nitric oxide synthase (NOS) mRNA, Complete cds. Accession AF062749.

Noble, W. C. (1998). Skin bacteriology and the role of *Staphylococcus aureus* in infection. *Bri. J. Dermotol.* **139**. 9-12.

Novick, R. P., Ross, H. F., Projan, S. J., *et al.* (1993). Synthesis of *Staphylococcus* virulence factors is controlled by a regulatory RNA molecule. *The EMBO. J.* **12.**3967-3975.

Nunokawa, Y., Ishida, N. and Tanaka, S. (1993). Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **191**. 89-95.
Rattus norvegicus mRNA for nitric oxide synthase, Complete cds. Accession D14051.

Ogura, T., Yokoyama, T., Fujisawa, H., *et al.* (1993). Structural diversity of neuronal nitric oxide synthase mRNA in the nervous system. *Biochem. Biophysi. Res. Commun.* **193**. 1014-1022.
Mouse mRNA for nitric oxide synthase, Complete cds. Accession D14552.

Olsson, C. and Holmgren, C. (1997). Nitric oxide in the fish gut. *Comp Biochem Physiol. A. Physiol.* **118**. 959-964.

Ortiz de Montellano, P. R., Nishida, C., Rodriguez-Crespo, I., *et al.* (1998). Nitric oxide synthase structure and electron transfer. *Drug Metab. Distribu.* **26**. 1185-1189.

Ott, S. R. and Burrows, M. (1998). Nitric oxide synthase in the thoracic ganglia of the locust: distribution in the neuropiles and morphology of neurones. *J. Comp. Neurol.* **395**. 217-230.

Oyan, A. M., Holmquist, B. and Goksoyr, A. (1998). Inducible nitric oxide synthase (iNOS) cDNA from Atlantic salmon. Unpublished.
Salmon salar inducible nitric oxide synthase mRNA, Partial cds. Accession F088999.

Palmer, R.M., Moncada, S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **158**.348-52.

Palumbo, A., Di Cosmo, A., Poli, A., *et al.* (1999). A calcium/calmodulin-dependent nitric oxide synthase, NMDAR2/3 receptor subunits, and glutamate in the CNS of the cuttlefish Sepia officinal is: localisation in specific neural pathways controlling the inking system. *J. Neurochem.* **73**. 1254-1263.

Parikh, A. and Guengerich, F. P. (1997). Expression, purification, and charac - terisation of a catalytically active human cytochrome P450 1A2:rat NADPH-cytochrome P450 reductase fusion protein. *Protein Exp. Purif.* **9**. 346-54.

Pearson, W.R., Lipman, D.J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U. S. A.* **85**.2444-8.

Perl, T.M. and Golub, J. E. (1998). New approaches to reduce *Staphylococcus aureus* nosocomial infection rates: treating *S.aureus* nasal carriage. *Ann. Pharmacother.* **32.** S7-S16.

Porteros, A., Arevalo, R., Crespo, C., *et al.* (1996). Nitric oxide synthase activity in the olfactory bulb of anuran and urodele amphibians. *Brain Res.* **724.** 67-72.

Poulos, T. L., Raman, C. S., Li, H. (1998). NO news is good news. *Structure* **6**. 255-8.

Presta, A., Siddhanta, U., Wu, C., *et al.* (1998). Comparative functioning of dihydro- and tetrahydrobiopterins in supporting electron transfer, catalysis, and subunit dimerisation in inducible nitric oxide synthase. *Biochemistry.* **37**. 298-310.

Projan, S. J., Kornblum, J., Moghazeh, L., *et al.* (1995). Comparative sequence and functional analysis of pT181 and pC221, cognate plasmid replicons from *Staphylococcus aureus. Mol. Gen. Genet.* **199.** 452-64.

Pufahl, R. A. and Marletta, M. A. (1993). Oxidation of NG-hydroxy-L-arginine by nitric oxide synthase- evidence for the involvement of the heme in catalysis. *Biochem. Biophys. Res. Commun.* **193**. 963-70.

Pufahl, R. A., Wishnok. J. S. and Marletta, M. A. (1995). Hydrogen peroxide-supported oxidation of N-hydroxy-L-arginine by nitric oxide synthase. *Biochemistry* **34**. 1930-1941.

Pullen, A. H., Humphreys, P. and Baxter, R. G. (1997). Comparative analysis of nitric oxide synthase immunoreactivity in the sacral spinal cord of the cat, macaque and human. *J. Anat.* **191**. 161-175.

Raman, C. S., Li, H.Y., Martasek, P., *et al.* (1998). Crystal structure of constitutive endothelial nitric oxide synthase: A paradigm for pterin function involving a novel metal center. *Cell.* **95**. 939-950.

Regulski, M. and Tully, T. (1995). Molecular and biochemical characterisation of dNOS: a Drosophila Ca+/calmodulin-dependent nitric oxide synthase. *Proc. Natl. Acad. Sci., U.S.A.* **92**. 9072-9076.
Drosophila melanogaster Ca/calmodulin-dependent nitric oxide synthase (NOS) mRNA, Complete cds. Accession U25117.

Ribeiro, E. A., Cunha, F.Q, Tamashiro, W. M., *et al.* (1999). Growth phase-dependent subcellular localisation of nitric oxide synthase in maize cells. *FEBS. Lett.* **445.** 283-286.

Richards, M. K.and Marletta, M. A. (1994). Characterisation of neuronal nitric oxide synthase and a C415H mutant, purified from a baculovirus overexpression system. *Biochemistry.* **33.** 14723-32.

Riefler G.M., and Firestein B.L. (2001). Binding of neuronal nitric oxide synthase to carboxy-terminal binding protein changes the localisation of carboxy-terminal protein from the nucleus to the cytosol. A novel function for targeting by the PDZ domain of neuronal nitric oxide synthase. JBC Papers in press. Published on October 5, 2001.

Riveros-Moreno, V., Heffernan, B., Torres, B., *et al.* (1995). Purification to omogeneity and characterisation of rat brain recombinant nitric oxide synthase. *Eur.J. Biochem.* **230**. 52-57.

Robbins, R. A., Grisham, M. B. (1997). Nitric oxide. *Int. J. Biochem. Cell. Biol.* **29**. 857-60.

Rodriguez-Crespo, I. and Oritiz de Montellano, P. (1996). Human endothelial nitric oxide synthase: expression in *Escherichia coli*, coexpression with calmodulin, and characterisation. *Arch. Biochem. Biophys.* **336**. 151-156.

Rodriguez-Crespo, I., Gerber, N. C., Ortiz de Montellano, P. R. (1996). Endothelial nitric oxide synthase: expression inn *Escherichia coli*, spectroscopic characterisation, and role of tetrahydrobiopterin in dimer formation. *J. Biol. Chem.* **271**. 11462-11467.

Roman, L. J., Sheta, E. A., Martasek, P., *et al.* (1995). High-level expression of functional rat neuronal nitric oxide synthase in *Escherichia coli. Proc. Natl. Acad. Sci., U.S.A.* **92**. 8428-8432.

Rondouin, G., Bockaert, J. and Lerner-Natoli, M. (1993). L-nitroarginine, an inhibitor of NO synthase, dramatically worsens limbic epilepsy in rats. *NeuroRep.* **4**. 1187-1195.

Ruettinger, R. T., Wen, L. P. and Fulco, A. J. (1989). Coding nucleotide, 5' regulatory, and deduced amino acid sequences of P-450BM-3, a single peptide cytochrome P-450:NADPH-P-450 reductase from Bacillus megaterium. *J. Biol. Chem.* **264**. 10987-95.

Rusche, K. M., Spiering, M. M. and Marletta, M. A. (1998). Reactions catalysed by tetrahydrobiopterin-free nitric oxide synthase. *Biochemistry.* **37**. 15503-15512.

Salerno, J. C., Martasek, P., Roman, L. J., *et al.* (1996). Electron paramagnetic resonance spectroscopy of the heme domain of inducible nitric oxide synthase: binding of ligands at arginine site induces changes in the heme ligation geometry. *Biochemistry.* **35**. 7626-7630.

Salter, M., *et al.* (1991). Measurement of NOS activity by citrulline assay. *FEBS.* **291**. 145-49.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, Second Edition. Cold Spring Harbor Laboratory Press, 1989.

Sari, M. A., Booker, S., Jaouen, M., *et al.* (1996). Expression in yeast and purification of functional macrophage nitric oxide synthase. Evidence for cysteine-194 as iron proximal ligand. *Biochemistry.* **35**. 7204-13.

Satoh, K., Arai, R., Ikemoto, K., *et al.* (1995). Distribution of nitric oxide synthase in the central nervous system of Macaca fuscata: subcortical regions. *Neurosci.* **66**. 685-696.

Scheinker, V., Stasiv, Y. and Enikolopov, G. (1998). Unpublished.

Xenopus laevis neuronal nitric oxide synthase mRNA, Complete cds. Accession AF053935.

Schmalix, W. A., Singh, J. J., Kapfhammer, P., *et al.* (1996). Stable expression of human inducible nitric oxide synthase in V79 Chinese hamster cells. *Biochem. Pharmacol.* **52.** 1365-74.

Schmidt, H. H. H. W., Smith, R. M., Nakane, M., *et al.* (1992). Ca2+/calmodulin-dependent NO synthase type I: a biopteroflavoprotein with Ca2+/calmodulin-independent diaphorase and reductase activities. *Biochemistry.* **31**. 3243-3249.

Schwan, W. R., Coulter, S. N., Wang, E. Y., *et al.* (1998). Identification and characterisation of the PutP proline permease that contributes to in vivo survival of *Staphylococcus aureus* in animal models. *Infect. Immun.* **66**. 567-72.

Schwemmer, M. and Bassenge, E. (1999). Assembly and characterisation of canine heart endothelial nitric oxide synthase cDNA and 5'-flanking sequence by homology (RT-) PCR cloning. *Nitric Oxide.* **3**. 254-264.
Canis familiaris nitric oxide synthase (NOS) mRNA, Complete cds. Accession AF143503.

Segal, S. S., Brett, S. E. and Sessa, W. C. (1999). Codistribution of NOS and caveolin throughout peripheral vasculature and skeletal muscle of hamsters. *Am. J. Physiol.* **277**. H1167-1177.

Serfozo, Z., Elekes, K. and Varga, V. (1998). NADPH-diaphorase activity in the nervous system of the embryonic and juvenile pond snail, *Lymnaea stagnlis. Cell Tis. Res.* **292**. 579-586.

Sessa, W. C., Harrison, J. K., Barber, C. M., *et al.* (1992). Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase. *J. Biol. Chem.* **267**. 15247-15276.

Bos taurus nitric oxide synthase mRNA, Complete cds. Accession M95674.

Sessa, W. C. (1994). The nitric oxide synthase family of proteins. *J. Vas. Res.* **31**. 131-143.

Shirato, M., Sakamoto, M., Uchida, Y., *et al.* (1998). Molecular cloning and characterisation of Ca2+-dependent inducible nitric oxide synthase from guinea pig lung. Unpublished.
Cavia porcellus inducible nitric oxide synthase (NOS) mRNA, Complete cds. Accession AF027180.

Smith, A. P. L. Unpublished.
S.scrofa mRNA for nitric oxide synthase, part cds. Accession Y11025.

Soderstrom, V., Hylland, P. and Nilsson, G. E. (1995). Nitric oxide synthase inhibitor blocks acetylcholine induced increase in brain blood flow in rainbow trout. *Neurosci. Lett.* **197**. 191-194.

Soderstrom, V., Nilsson, G. E. and Lutz, P. L. (1997). Effects of inhibition of nitric oxide synthesis and of hypercapnia on blood pressure and brain blood flow in the turtle. *J. Exp. Biol.* **200**. 815-820.

Southan, G. J. and Szabo, C. (1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem. Pharmacol.* **51**. 383-394.

Stachura, J., Konturek, J. W., Karcezewska, A., *et al.* (1996). *Helicobacter pylori* from duodenal ulcer patients expresses inducible nitric oxide synthase immuno-reactivity in vivo and in vitro. *J. Physiol. Pharmacol.* **47**. 131-135.

Stenger, S., Thuring, H., Rollinghoff, M., *et al.* (1995). L-N- (1-Iminoethyl)-lysine potent inhibits inducible nitric oxide synthase and is superior to N-monomethyl-arginine in vitro and in vivo. *Eur. J. Pharmacol.* **294**. 703-712.

Stranden, A. M., Ehler, K., Labischinski, H., *et al.* (1997). Cell wall monoglycine cross-bridge and methicillin hypersusceptibility in a *femAB* null mutant of methicillin-resistant *Staphylococcus aureus. J. Bacteriol.* **179**. 9-16.

Stuehr, D. J. and Nathan, C. F. (1989). Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. *J. Experi. Med.* **169**. 1543-55.

Stuehr, D. J., Kwon, N. S. and Nathan, C. F., *et al.* (1991). N-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine. *J. Biol. Chem.* **266**. 6259-6263.

Stuehr, D. J. (1997). Structure-function aspects in the nitric oxide synthases. *Annu. Rev. Pharmacol. Toxicol.* **37**. 339-359.

Su, C., Kanevsky, I., Jayarao, B. M., *et al.* (1998). Phylogenetic relationships of *Staphylococcus aureus* from bovine mastitis based on coagulase gene polymorphism. *Vet Microbiol.* **71**. 53-58.

Sulavik, M. C. and Barg, N. L. (1998). Examination of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* mutants with low-level fluoroquinolone resistance. *Antimicrb. Agent. Chemother.* **42**. 3317-19.

Thomsen, L. L., Scott, J. M. J., Topley, P., *et al.* (1997). Selective inhibition of inducible nitric oxide synthase inhibits tumor growth in vivo: studies with 1400W, 1 novel inhibitor. *Cancer Res.* **57**. 3300-3304.

Tierney, D. L., Huang, H., Martasek, P., *et al.* (1999). ENDR spectroscopic evidence for the position and structure of N-hydroxy-L-arginine bound to holo-neuronal nitric oxide synthase. *Biochemistry.* **38**. 3704-3710.

Titheradge, M. A. Nitric oxide protocols Methods in molecular biology, Volume 100. Humana press. 1998.

Tochio, H., Zhang, Q., Mandal, P., *et al.* (1999). Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide. *Nature Struct. Biol.* **6**. 417-421.

Trieu-Cuot, P., Gerbaud, G., Lambert, T., *et al.* (1985). *In vivo* transfer of genetic information between Gram-positive and Gram-negative bacteria. *The EMBO. J.* **4**. 3583-87.

Tseng, H. C., Lin, C. K., Hsu, B. J., *et al.* (1990). The melanin operon of *Streptomyces antibioticus*: expression and use as a marker in Gram-positive bacteria. *Gene.* **86.** 123-28.

Venema, R. C., Ju, H., Zou, R., *et al.* (1997). Subunit interaction of endothelial nitric-oxide synthase. Comparisons to the neuronal and inducible nitric oxide synthase isoforms. *J. Biol. Chem.* **272**. 1276-1282.

Vizzard, M. A., Erickson, K. and de Groat, W. C. (1997). Localisation of NADPH diaphorase in the thoracolumbar and sacrococcygeal spinal cord of the dog. *J. Auton. .Nerv. Syst.* **64**. 128-142.

Vorherr, T., Knopfel, L., Hofmann, F., *et al.* (1993). The calmodulin binding domain of nitric oxide synthase and adenylyl cyclase. *Biochemistry.* **32**. 6081-6088.

Wang, J., Stuehr, D. J. and Rousseau, D. L. (1997). Interactions between substrate analogues and heme ligands in nitric oxide synthase. *Biochemistry.* **36**. 4595-4606.

Wang, M., Roberts, D. L., Paschke, R., *et al.* (1997). Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc. Natl. Acad. Sci., U.S.A.* **94**. 8411-8416.

Wang, X., McGregor, C. G. A. and Miller, V. M. (1998). Induction and cDNA sequence of inducible nitric oxide synthase from canine aortic smooth muscle cells. Unpublished.
Canis familiaris inducible nitric oxide synthase mRNA, Complete cds. Accession AF077821.

White, K. A. and Marletta, M. A. (1992). Nitric oxide synthase is a cytochrome P450 type hemoprotein. *Biochemistry.* **31**. 6627-6631.

Witteveen, F. B., Giovanelli, J., Yim, M. B., *et al.* (1998). Reactivity of the flavin semiquinone of nitric oxide synthase in the oxygenation of arginine to N-hydroxyarginine, the first step of nitric oxide synthesis. *Biochem. Biophysiol. Res. Commun.* **250**. 36-42.

Wolff, D. J., Datto, G. A., Samatovicz, R. A., *et al.* (1993a). Calmodulin-dependent nitric-oxide synthase, mechanism of inhibition by imidazole and phenylimidazoles. *J. Biol. Chem.* **268**. 9425-29.

Wolff, D. J., Datto, G. A. and Samatovicz, R. A. (1993b). The dual mode of inhibition of calmodulin-dependent nitric-oxide synthase by antifungal imidazole agents. *J. Biol. Chem.* **268**. 9430-36.

Wolff, D. J. and Gribin, B. J. (1994a). Interferon-γ-inducible murine macrophage nitric oxide synthase: studies on the mechanism of inhibition by imidazole agents. *Arch. Biochem. Biophysic.* **311**. 293-99.

Wolff, D. J., Lubeskie, A. and Umansky, S. (1994b). The inhibition of the constitutive bovine endothelial nitric oxide synthase by imidazole and indazole agents. *Arch. Biochem. Biophys.* **314**. 360-66.

Wolff, D. J., Lubeskie, A., Gauld, D. S, *et* al. (1998). Inactivation of nitric oxide synthase and cellular nitric oxide formation by N-iminoethyl-L-lysine and N-iminoethyl-L-ornithine. *Eur. J. Pharmacol.* **350**. 325-334.

Wu, C., Zhang, J., Abu-Soud, H., *et al.* (1996). High-level expression of mouse inducible nitric oxide synthase in *Escherichia coli* require coexpression with calmodulin. *Biochem. Biophys. Res. Commun.* **222**. 439-444.

Wu, S., De Lencastre, H. and Tomasz, A. (1996). Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing. *J. Bacteriol.* **178**. 6036-6042.

Xie, Q. W., Cho, H. J., Calaycay, J., *et al.* (1992). Cloning and characterisation of inducible nitric oxide synthase from mouse macrophages. *Science.* **256**. 225-8.

Xu, W. M., Charles, I. G., Liu, L. Z., *et al.* (1995). Molecular genetic analysis of the duplication of human inducible nitric oxide synthase (NOS2) sequences. *Biochem. Biophys. Res. Commun.* **212**. 466-472.

Xu, W. M., Charles, I. G., Liu, L. Z., *et al.* (1996). Molecular cloning and structural organisation of the human inducible nitric oxide synthase gene (NOS2). *Biochem. Biophys. Res. Commun.* **219.** 784-788.

Xu, W. M., Liu, L. Z., Emson, P. C., *et al.* (1997). Evolution of a homopurine-homopyrimidine pentanucleotide repeat sequence upstream of the human inducible nitric oxide synthase gene. *Gene.* **204.** 165-170.

Xu, W. M., Liu, L. Z. (1998). Nitric oxide: from a mysterious labile factor to the molecule of the Noble Prize. *Cell Res.* **8**. 251-258.

Yuda, M. Unpublished.
Rhodnius prolixus salivary gland nitric oxide synthase mRNA, Complete cds. Accession U59389.

Zhang, J. and Snyder, S. H. (1995). Nitric oxide in the nervous system. *Ann. Rev. Pharmacol. Toxicol.* **35**. 213-233.

Zhang, J. L., Patel, J. M. and Block, E. R. (1996). Molecular cloning, characterisation and expression of a nitric oxide synthase from porcine pulmonary artery endothelial cells. Unpublished.
Sus scrofa nitric oxide synthase (NOS) mRNA, Complete cds. Accession U59924.

Zielinski, B. S., Osahan, J. K., Hosseini, T. J., *et al.* (1996). Nitric oxide synthase in the olfactory mucosa of the larval sea lamprey (*Petromyzon marinus*). *J. Comp. Eurol.* **365**. 18-26.

APPENDIX

CONTENTS

Appendix 1. GeneBank data for *yflM*

LOCUS C69811 336 aa BCT 15-OCT-1999

DEFINITION nitric-oxide synthase homolog *yflM* - *Bacillus subtilis.*

ACCESSION C69811

PID g7475678

VERSION C69811 GI:7475678

DBSOURCE pir: locus C69811;

summary: #length 336 #molecular-weight 38750 #checksum 3170;

genetic: #gene *yflM*;

PIR dates: 05-Dec-1997 #sequence_revision 05-Dec-1997

text_change 15-Oct-1999.

KEYWORDS .

SOURCE *Bacillus subtilis.*

ORGANISM *Bacillus subtilis*

Bacteria; Firmicutes; Bacillus/Clostridium group;

Bacillus/Staphylococcus group; Bacillus.

REFERENCE 1 (residues 1 to 336)

AUTHORS Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Boriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Codani, J.J., Connerton, I.F., Cummings, N.J., Daniel, R.A., Denizot, F., Devine, K.M., Duesterhoeft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger,D., Fritz,C., Fujita,M., Fujita,Y., Fuma,S., Galizzi,A., Galleron,N., Ghim,S.Y., Glaser,P., Goffeau,A., Golightly,E.J., Grandi,G., Guiseppi,G., Guy,B.J., Haga,K., Haiech,J., Harwood,C.R., Henaut,A., Hilbert,H., Holsappel,S., Hosono,S., Hullo,M.F., Itaya,M., Jones,L., Joris,B., Karamata,D., Kasahara,Y., Klaerr-Blanchard,M., Klein,C., Kobayashi,Y., Koetter,P., Koningstein,G., Krogh,S., Kumano,M., Kurita,K., Lapidus,A., Lardinois,S., Lauber,J., Lazarevic,V., Lee,S.M., Levine,A., Liu,H., Masuda,S., Maueel,C., Medigue,C., Medina,N., Mellado,R.P., Mizuno,M., Moestl,D., Nakai,S., Noback,M., Noone,D., O'Reilly,M., Ogawa,K., Ogiwara,A., Oudega,B., Park,S.H., Parro,V., Pohl,T.M., Portetelle,D., Porwolik,S.,

Prescott,A.M., Presecan,E., Pujic,P., Purnelle,B., Rapoport,G., Rey,M., Reynolds,S., Rieger,M., Rivolta,C., Rocha,E., Roche,B., Rose,M., Sadaie,Y., Sato,T., Scanlon,E., Schleich,S., Schroeter,R., Scoffone,F., Sekiguchi,J., Sekowska,A., Seror,S.J., Serror,P., Shin,B.S., Soldo,B., Sorokin,A., Tacconi,E., Takagi,T., Takahashi,H., Takemaru,K., Takeuchi,M., Tamakoshi,A., Tanaka,T., Terpstra,P., Tognoni,A., Tosato,V., Uchiyama,S., Vandenbol,M., Vannier,F., Vassarotti,A., Viari,A., Wambutt,R., Wedler,E., Wedler,H., Weitzenegger,T., Winters,P., Wipat,A., Yamamoto,H., Yamane,K., Yasumoto,K., Yata,K., Yoshida,K., Yoshikawa,H.F., Zumstein,E., Yoshikawa,H. and Danchin,A.

TITLE The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*

JOURNAL Nature 390 (6657), 249-256 (1997)

MEDLINE 98044033

FEATURES Location/Qualifiers

source 1..336

/organism="Bacillus subtilis"

/db_xref="taxon:1423"

Protein 1..336

/product="nitric-oxide synthase homolog *yflM*"

ORIGIN

```
     1 mkdrladiks eidltgsyvh tkeelehgak mawrnsnrci grlfwnslnv
    51 idrrdvrtke evrdalfhhi etatnngkir ptitifppee kgekqveiwn
   101 hqliryagye sdgerigdpa scsltaacee lgwrgertdf dllplifrmk
   151 gdeqpvwyel prslvievpi thpdieafsd lelkwygvpi isdmklevgg
   201 ihynaapfng wymgteigar nladekrydk lkkvasvigi aadyntdlwk
   251 dqalvelnka vlhsykkqgv sivdhhtaas qfkrfeeqee eagrkltgdw
   301 twlippispa athifhrsyd nsivkpnyfy qdkpye
```

Appendix 2. BLAST result for SANOS

```
TBLASTN 2.0MP-WashU [27-Jan-2000] [linux-x86 13:06:01 27-Jan-2000]
Copyright (C) 1996-2000 Washington University, Saint Louis, Missouri USA.
All Rights Reserved.
Reference:  Gish, W. (1996-1999) http://blast.wustl.edu
Notice:  statistical significance is estimated under the assumption that the
equivalent of one entire reading frame of the database codes for protein and
that significant alignments will involve only coding reading frames.
Query=
        (550 letters)
Database:  /usr/local/db/s_aureus
           71 sequences; 2,745,314 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                      Smallest
                                                                        Sum
                                                    Reading  High  Probability
Sequences producing High-scoring Segment Pairs:       Frame Score  P(N)       N

8086                                                    -3   712  1.1e-70   1

>8086
     Length = 49,954

  Minus Strand HSPs:

 Score = 712 (255.7 bits), Expect = 1.1e-70, P = 1.1e-70
 Identities = 148/356 (41%), Positives = 208/356 (58%), Frame = -3

Query:   139 LLPQAIEFVNQYYGSFKEAKIEEHLA--RVEAVTKEIETTGTYQLTGDELIFATKQAWRN 196
             L  +A  F+   Y   KE   E  +   R+  +  EI+ TGTY  T +ELI+  K AWRN
Sbjct: 15053 LFKEAQAFIENMY---KECHYETQIINKRLHDIELEIKETGTYTHTEEELIYGAKMAWRN 14883

Query:   197 APRCIGRIQWSNLQVFDARSCSTAREMFEHICRHVRYSTNNGNIRSAITVFPQRSDGKHD 256
             + RCIGR+ W +L V DAR  +         I  H+  +TN G ++  IT++  + DG
Sbjct: 14882 SNRCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPYITIYAPK-DGP-- 14712

Query:   257 FRVWNAQLIHYAGYQMPDGSIRGDPANVEFTQLCIDLGWKPKYGRFDVVPLVLQANGRDP 316
              +++N QLI YAGY   D    GDPA  E T+L   LGWK K   FDV+PL+ Q
Sbjct: 14711 -KIFNNQLIRYAGY---DNC--GDPAEKEVTRLANHLGWKGKGTNFDVLPLIYQLPNESV 14550

Query:   317 ELFEIPPDLVLEVAMEHPKYEWFRELELKWYALPAVANMLLEVGGLEFPGCPFNGWYMGT 376
             + +E P  L+ EV +EH  Y   R+L LKWYA+P ++NM L++GG+ +P  PFNGWYM T
Sbjct: 14549 KFYEYPTSLIKEVPIEHNHYPKLRKLNLKWYAVPIISNMDLKIGGIVYPTAPFNGWYMVT 14370

Query:   377 EIGVRDFCDVQRYNILEEVGRRMGLETHKLASLWKDQAVVEINIAVLHSFQKQNVTIMDH 436
             EIGVR+F D  RYN+LE+V      +T K  S  KD+A+VE+N AV HSF+K+ V+I+DH
Sbjct: 14369 EIGVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAVYHSFKKEGVSIVDH 14190

Query:   437 HSTAESFMKYMQNEYRSRGGCPADWIWLVPPMSGSITPVFHQEMLNYVLSPFYYYQ 492
              + A+ F  + +NE +        W WL PP+S ++T  +H    N V  P ++Y+
Sbjct: 14189 LTAAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDNTVKDPNFFYK 14022

Parameters:
  B=5
  ctxfactor=6.00
  E=10
  Query                        -----  As Used  -----     -----  Computed  ----
  Frame  MatID Matrix name     Lambda    K       H       Lambda    K       H
   +0      0   BLOSUM62        0.320   0.136   0.427     same     same     same
               Q=9,R=2         0.244   0.0300  0.180      n/a      n/a      n/a

 Query
  Frame  MatID  Length  Eff.Length     E    S W   T  X   E2     S2
   +0      0      550       550       10.  61 3  13 22  0.071   37
                                                 35  0.063   42

Statistics:

  Database:  /usr/local/db/s_aureus
    Title:  /usr/local/db/s_aureus
    Release date:  unknown
```

```
    Posted date:  3:35:02 PM EDT Oct 10, 2000
    Last modified:  unknown
    First created:  unknown
    Format:  BLAST-1.4
  # of letters in database:  2,745,314
  # of sequences in database:  71
  # of database sequences satisfying E:  1
  No. of states in DFA:  560 (60 KB)
  Total size of DFA:  124 KB (128 KB)
  Time to generate neighborhood:  0.01u 0.00s 0.01t  Elapsed: 00:00:00
  No. of threads or processors used:  1
  Search cpu time:  2.48u 0.01s 2.49t  Elapsed: 00:00:03
  Total cpu time:  2.50u 0.01s 2.51t  Elapsed: 00:00:03
  Start:  Tue Nov  7 08:14:28 2000   End:  Tue Nov  7 08:14:31 2000

The top-scoring match came from this contig (up to 1000bp on either side of
the hit are shown):

>8086 (from 13022 to 16053)
CTTGGACGAGTGGTAACGAAACGTATACCGCAGCATCGTGTAAAAACAATACAAACAAAA
GAAAGTCAACCAAGGATGGATTCCTATTTTAATCCTTGGTTGACTCTTTATTTTATTTAA
ATTGTAGAACCTAGAAAATAAAGTTTAATTAAAAGCACCAATCATTTCTACTTTGAAATC
TAAGGTTTCTAAAATAGCAATGACTTTCTTTATATCGGTTGTAATTGCAGAATCAGCCTG
AACGAAAAATCGATACATACCTAATTGTGTTTTTAAAGGACGAGACTCAATCCAGGATAA
ATTAATATTAAACAAAGCAAATGTATTAAGCACACTTGCTAACAACCCAGGTTTATCATG
CATTGGTGTAATTAAAAACATCAATGATGTCGCATTTTGATCAAATTGCTGCTGATTTTT
TATAACTAAAAAACGTGTCACGTTATGTGGATAGTCTTCAATATGTGTATCAATAGGTGT
AAAACCATAAGCTTCGCCACTACCTAAAGGTGCAATTGCTGCAACGCCATTTTCAATTTT
AGTCAAACTTTGAATTGTACTGTCGACATAATCATAGTCAAATTGATGTTGATGTATGTA
ATTTGTTGTTTGACTGATTGCTGGTGCAATAGAATACACTTTTTTAATATCAGAAATGGA
ATCTGTTCCATTACCATATAATGCAAAGTTAATATCTAAACGTATTTCACCGTGTGCAAA
GACATCTTGCTGTGCAAGTGCATCTGCCACAATGTTGATTGTTCCTTCTATAGAATTTTC
AATAGGGACAACACCAATCGATGTGTCATCATCTGCAACTGCCTTGATGACTTCAAAAAA
ATTTGACTTTGGTTGAAAAGTTGCTTCATTTTCAGAAAAATACTGACGACAAGCCAAATA
TGAAAATGTACCTTTAGGGCCTAAATAATATAATTGCATATGCTACACCTCTACTAACTT
AATGATGGAAAGGGCACTGGTTAGCATTTGATTCTTTCTTTTTATAGAAAAAGTTTGGAT
CTTTTACTGTATTGTCATATCCGTGATGATAATTTGACGTCAATGTTGGAGATAATGGCG
GTGCTAGCCAAGACCATTTTCCGGTAACTTGACGACCTTGTTGTGCTTCGTTACGTTCGA
ATAGTTCGAATTGCTTTGCAGCGGTCAAATGATCGACAATTGATACGCCTTCTTTTTTAA
AGGAATGATACACAGCATAGTTCAATTCAACAAGTGCTCGATCTTTATTAAATGAATTAT
TTTTAAGTGTATCAAATTCAAACGCATCTGCAACTTTTTCTAGTAAATTGTAACGGTAAT
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TTAATGAAGTAGGATATTCGTAAAATTTAACTGACTCATTAGGTAATTGGTAAATCAGTG
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CTTCTTTTTCAGCAGGATCACCACAATTGTCATAGCCAGCATAGCGAATTAATTGATTGT
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CATTTGTAGCCTGTGTAATATGATAAGTAATTGATGATAAGAACGATGCTTCGTCAGTAA
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AAAGGCTAGCAGAATTCGTCAAATTGCATCAAATGATAAATTAATGGAGTTTGGTACACG
TCGTGCGCAAGAAATTGATGCAGCATTGTGGGGCGCTAGAGCTGCTTACATCGGGGGCTT
TGATTCTACAAGTAATGTTAGGGCGGGGAAATTATTTGGTATACCTGTGTCTGGTACACA
TGCACATGCATTTGTCCAAACTTATGGAGACGAATATGTTGCCTTCAAAAAATATGCTGA
AAGACATAAAAATTGTGTGTTCCTAGTAGATACATTCCATACTTTAAAATCTGGCGTGCC
AAATGCAATAAAAGTTGCAAAAGAATTAGGTGACAAAATTAACTTTGTAGGTATTCGATT
AGATTCTGGAGATATCGCTTATTTATCTAAAG
```

Appendix 3. BLAST result for STAPHRED

Reference: Gish, W. (1996-1999) http://blast.wustl.edu

Notice: statistical significance is estimated under the assumption that the equivalent of one entire reading frame of the database codes for protein and that significant alignments will involve only coding reading frames.

Query=
(653 letters)

Database: /usr/local/db/s_aureus
71 sequences; 2,745,314 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

Sequences producing High-scoring Segment Pairs:	Reading Frame	High Score	Smallest Sum Probability P(N)	N
8076	-2	365	5.7e-39	3
8104	+2	72	0.99	1

>8076
Length = 309,573

Minus Strand HSPs:

Score = 365 (133.5 bits), Expect = 5.7e-39, Sum P(3) = 5.7e-39
Identities = 97/305 (31%), Positives = 159/305 (52%), Frame = -2

```
Query:    326 QALTYFLDITTPPTQLLLQKLAQVATEEPERQRLEALCQPSEYSKWKFTNSPTFLEVLEE 385
              +ALT   + T     LL  K A +  +  E   L    Q   +++ ++  +  F++++ +
Sbjct: 139148 EALTSHFEFTKLTLPLL--KNADIYFDNEE---LSERIQDESWAR-EYVINRDFIDLITD 138987

Query:    386 FPSLRVSAGFLLSQLPILKPRFYSISSSRDHTPTEIHLTVAVVTYHTRDGQGPLHHGVCS 445
              FP++ +    +   L  L PR YSISSS   TP E+H+TV  V Y      G    GVCS
Sbjct: 138986 FPTIELQPENMYQILRKLPPREYSISSSFMATPDEVHITVGTVRYQAH---GRERKGVCS 138816

Query:    446 T-WLNSLKPQDPVPCFVRNASGFHLPEDPSHPCILIGPGTGIAPFRSFWQQRLHDSQHKG 504
               +   +KP D VP +++    F  P     P I+IGPGTGIAPFR++ Q+R    +  G
Sbjct: 138815 VHFAERIKPGDIVPIYLKKNPNFKFPMKQDIPVIMIGPGTGIAPFRAYLQER----EELG 138648

Query:    505 VRGGRMTLVFGCRRPDEDHIYQEEMLEMAQKGVLHAVHTAYSRLPGKPKVYVQDILRQQL 564
              + G +  L FG +    D +Y+EE+ E  + G L  V  A+SR   + K YVQ  + ++
Sbjct: 138647 MTG-KTWLFFGDQHRSSDFLYEEEIEEWLENGNLTRVDLAFSR-DQEHKEYVQHRIMEE- 138477

Query:    565 ASEVLRVLHKEPGHLYVCGDVR-MARDVAHTLKQLVAAKLKLNEEQVEDYFFQLKSQKRY 623
               S+      ++   +Y+CGD + MA+DV   +K ++  +  +++E+ E    Q+K Q+RY
Sbjct: 138476 -SKRFNEWIEQGAAIYICGDEKCMAKDVHQAIKDVLVKERHISQEEAELLLRQMKQQQRY 138300

Query:    624 HEDIF 628
               D++
Sbjct: 138299 QRDVY 138285
```

Score = 142 (55.0 bits), Expect = 5.7e-39, Sum P(3) = 5.7e-39
Identities = 43/141 (30%), Positives = 68/141 (48%), Frame = -2

```
Query:     34 ASRVRVTILFATETGKSEALAWDLGALFSCAFNPKVVC-MDKYRLSCLEEERLLLVVTST 92
              A++  VT+L+ +E+G +  LA       S   +  V+  MD+Y  + + +   L ++TST
Sbjct: 139913 ANQRHVTVLYGSESGNAMRLAEIFSERLSDIGHQVVLMSMDEYDTTNIAQLEDLFIITST 139734

Query:     93 FGNGDCPGNGEKLKKSLFMLKELN-NKFRYAVFGLGSSMYPRFCAFAHDIDQKLSHLGAS 151
               G G+ P N     + L       N N  RY+V  LG   Y  FC    D+D  L +LGA
Sbjct: 139733 HGEGEPPDNAWDFFEFLEDDNAPNLNHVRYSVLALGDQTYEFFCQAGKDVDVLLENLGAE 139554

Query:    152 QLTPMGEGDELSGQEDAFRSW 172
              ++    + D +  +EDA   W
Sbjct: 139553 RICKRVDCD-IDYEEDA-EKW 139497
```

Score = 60 (26.2 bits), Expect = 5.7e-39, Sum P(3) = 5.7e-39

```
 Identities = 17/70 (24%), Positives = 33/70 (47%), Frame = -2

Query:    220 LSKALSSMHAK-NVFTMRLKSRQNLQSPTSSRATILVELSCEDGQGLNYLPGEHLGVCPG 278
              +  A    ++K N +   + +  NL    S++ T  +E   +D    +Y PG+ +   P
Sbjct: 139424 IKSAKEKKYSKSNPYQAEVLANINLNGTDSNKETRHIEFLLDDFSE-SYEPGDCIVALPQ 139248

Query:    279 NQPALVQGIL 288
              N P LV+ ++
Sbjct: 139247 NDPELVEKLI 139218

 Score = 52 (23.4 bits), Expect = 6.2e-30, Sum P(2) = 6.2e-30
 Identities = 16/39 (41%), Positives = 21/39 (53%), Frame = -1

Query:    294 GPTPHQTVRLEA---LDESGSY--WVSDKRLPPCSLSQA 327
              GPTP  TV L A   LD +  Y  W S   + PC+ S++
Sbjct: 272238 GPTPVPTVTLPAIGLLDVTNGYVPW-SISNITPCAPSKS 272125

 Score = 48 (22.0 bits), Expect = 2.3e-05, Sum P(3) = 2.3e-05
 Identities = 13/62 (20%), Positives = 30/62 (48%), Frame = -1

Query:    558 DILRQQLASEVLRVLHKEPGHLYVCGDVRMARDVAHTLKQLVAAKLKLNEEQVEDYFFQL 617
              DI + ++ +E   +  +  GHL   G +   R+  + L  +   + K N + ++D +  +
Sbjct: 121650 DIEKAKVINEEFEISKQFWGHLVKSGSIENPREFINPLPHISYVRGKNNVKFLKDRYEAM 121471

Query:    618 KS 619
              K+
Sbjct: 121470 KA 121465

 Score = 45 (20.9 bits), Expect = 4.3e-05, Sum P(3) = 4.3e-05
 Identities = 9/23 (39%), Positives = 15/23 (65%), Frame = -2

Query:    526 QEEMLEMAQKGVLHAVHTAYSRL 548
              +E M+  A +GVL+ ++T Y  L
Sbjct: 14441 KEHMIRAALEGVLYNLYTVYLAL 14373

 Score = 44 (20.5 bits), Expect = 3.7e-05, Sum P(2) = 3.7e-05
 Identities = 10/26 (38%), Positives = 13/26 (50%), Frame = -3

Query:    261 DGQGLNYLPGEHLGVCPGNQPALVQG 286
              D   L YL  + LGV P +  A + G
Sbjct: 114496 DSARLQYLISQELGVAPSSVDASIIG 114419

 Score = 43 (20.2 bits), Expect = 6.7e-05, Sum P(3) = 6.7e-05
 Identities = 15/51 (29%), Positives = 24/51 (47%), Frame = -3


Query:    357 QRLEALCQPSEYSKWKFTNSPTFLEVLEEFPSLRVSAGFLLSQLPILKPRF 407
              +RL  LC+      W F +     E+  +F S R   G LL Q  +L+ ++
Sbjct: 134008 KRLSLLCE------WWFHSLL*VSEIATQFESTRSGMGLLL*QSWLLRYKY 133874

 Score = 42 (19.8 bits), Expect = 5.9e-05, Sum P(2) = 5.9e-05
 Identities = 6/11 (54%), Positives = 8/11 (72%), Frame = -3

Query:    194 IPKLYTSNVTW 204
              +PK Y SN+ W
Sbjct: 21853 LPKTYLSNIEW 21821

 Score = 42 (19.8 bits), Expect = 5.9e-05, Sum P(2) = 5.9e-05
 Identities = 12/22 (54%), Positives = 13/22 (59%), Frame = -1

Query:    262 GQGLNYLPGE--HLGVCPGNQP 281
              G+G  YL GE  HLG   G QP
Sbjct: 65382 GKGTKYLLGEIKHLG--EGYQP 65323


>8104
     Length = 25,517

  Plus Strand HSPs:

 Score = 72 (30.4 bits), Expect = 4.2, P = 0.99
 Identities = 25/96 (26%), Positives = 44/96 (45%), Frame = +2

Query:    519 PDEDHIYQEEMLEMAQK-GVLHAVHTAYSRLPGKPKVYVQDILRQQLASEVLRVLHKEPG 577
```

```
            P+E     EE L +  + G  HA    YS+  G P   ++D +   +  E L+ L+K+ G
Sbjct: 14900 PNESEEQFEETLTLYDEVGFEHAYTYLYSQRDGTPAAKMKDNVPLNVKKERLQRLNKKVG 15079

Query:   578 HLYVCGDVRMARDVAHTLKQLVAAKLKLNEEQVEDY 613
            H Y    + M++    T+  L     K +++ +  Y
Sbjct: 15080 H-Y--SQIAMSKYEGQTVTVLCEGSSKKDDQVLAGY 15178

Parameters:
  B=5
  ctxfactor=6.00
  E=10

  Query                        -----  As Used  -----    -----  Computed  ----
  Frame  MatID Matrix name     Lambda    K       H      Lambda    K       H
   +0      0   BLOSUM62        0.320   0.136   0.413    same    same    same
               Q=9,R=2         0.244   0.0300  0.180     n/a     n/a     n/a

  Query
  Frame  MatID  Length  Eff.Length     E    S W   T  X   E2     S2
   +0      0      653       653       10.  62 3  13 22  0.071   37
                                                 36  0.063   42

Statistics:

  Database:  /usr/local/db/s_aureus
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  Total cpu time:  2.69u 0.01s 2.70t  Elapsed: 00:00:08
  Start:  Thu Nov 16 08:07:46 2000   End:  Thu Nov 16 08:07:54 2000
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1000bp on either side of
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GTGCTTGATAACGAACCGTACCAACGGTAATATGCACTTCATCTGGCGTTGCCATAAAAC
TACTAGAAATCGAATACTCTCTTGGTGGTAATTTTCTAAGGATTTGATACATATTCTCAG
```

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TTTCTTTATTTGAATCGGTACCATTTAAATTGATATTCGCTAATACTTCTGCTTGGTATG
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CACTTTGAATACCTTCTGATGTGGTATCAATAATATTAATGATGTCTGCCATCCACTTTT
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TGTTAATCTCTGTGACTTGTTTTTCTGTAAATGGACTATTAGAT
</body></html>

Appendix 4 SANOS crystallisation

(The experiment described here is from Dr. Dave Stammers' lab at Oxford).
2 mg aliquots of purified protein were stored at -80°C. The protein was defrosted in the presence of 2 mM L-arginine and concentrated to 10 mg/ml. Gel filtration was performed at 21°C in the absence of ligands to remove aggregated protein. The column was equilibrated with crystallisation buffer: 25 mM Tris pH7.4, 50mM NaCl and 1 mM DTT. The peak fractions were pooled and concentrated to 10 mg/ml and used immediately for crystallisation.

The sitting drop vapour diffusion method was used at 21°C using fresh gel-filtered and concentrated protein. The protein concentration was adjusted to 8 mg/ml by adding 5 mM SEITU and 0.3 M Non-detergent sulphobetaine 195. A final concentration of 1-2% γ-butyrolactone was added to the drop on setting up. Crystals were typically obtained from 100 mM Tris pH7.4, 3-9% PEG 6000, 5% MPD. The crystal used for structure determination grew from a drop with 10 mM $NADH_2$ as an additional additive.

Introduction

The crystal structure of two murine inducible NOS heme domains (amino acids 114 to 498, Crane, B.R., *et al.*, 1997; amino acids 66 to 498, Crane, B.R., *et al.*, 1998), have been recently published. Structures have also been reported for the bovine endothelial NOS heme domain (39 to 482 amino acids, Raman, C.S., *et al.*, 1998), human endothelial NOS heme domain (66 to 492 amino acids) and human inducible NOS heme domain (1 to 508 amino acids, Fischmann T.C., *et al.*, 1999).

Interestingly, the structure of the 114-498 iNOS heme domain is a monomer while the structure of the 66-498 construct is a dimer. These results parallel the catalytic activity, (Ghosh, D.K., *et al.*, 1997), demonstrating that the 114-498 constuct is inactive, while the 66-498 protein is active. Research on the eNOS crystal structure, (Raman, C.S., *et al.* 1998) identified a phylogenetically conserved Cys-(X)4-Cys

motif and its strategic location establish a structural role for the metal centre in maintaining the integrity of the BH_4 binding site (Raman, C.S., *et al.*, 1998).

The study of the crystal structures of NOS isoforms not only shows the unusual fold of the N-terminal heme domain compared to other proteins, but also highlights the atomic environment for necessary for stabilisation of activated oxygen intermediates key for catalysis. These studies have also identified key residues important in inhibor binding, and these will be useful for the design of compounds to enhance or inhibit NOS activity.

Overall structure of SANOS and the dimer interface

SANOS can be aligned between residues 133 to 499 in murine iNOS, and 125 to 491 in bovine eNOS. The crystal structure shows SANOS to be a dimer.

Two additional fragments are present at the N-terminus of the eukaryotic NOS, where they form part of the so-called 'N-terminal hook' (Crane, B.R., *et al.*, 1998). The hook seems to be absolutely required for dimer stability in the eukaryotic NOS (Chen, P.F., *et al.*, 1995). Two cysteines from each hook tetrahedrally co-ordinate a zinc ion to form a zinc-tetrahiolate at the dimer interface, additional contacts are made by this region with the pterin binding site and with the other subunit (Raman, C.S., *et al.*, 1998).

The lack of the 'hook region' in SANOS results in a major structural difference between SANOS and other NOS dimers. The cleft between the two monomers is wider, resulting in the site equivalent to the eukaryotic NOS BH_4 binding site being more open to the solvent.

BH_4 is not present in prokarotes and there is not N-terminal hook region in SANOS, to our surprise, a NAD appears in the interface of two SANOS monomers in its dimer structure, which might be functionally significant.

In eukaryotic NOS, the subunit interface ligand-binding site binds BH_4. BH_4 has been reported to have dual roles in both stabilising the dimer, and in participating in catalysis (Raman, C.S., *et al.*, 1999). While in SANOS the overall topology of the interface ligand-binding site is the same as that observed previously for mammalian NOS structures, there are some key differences.

In contrast to mammalian NOS, BH_4 has no stimulatory effect on SANOS binding to L-arginine, or in the catalytic conversion of NOHA to NO.

The heme site is buried in the interior of each monomer (Fig I). The residues in the haem binding motif that contact the haem in the mammalian NOS structures are conserved in SANOS, with heme making extensive contacts with the side-chains of Trp56, Arg51, Cys62, Pro104, Phe222, Asn223, Gly224, Trp225, Met227, Glu230, Trp316, Phe342 and Tyr344.

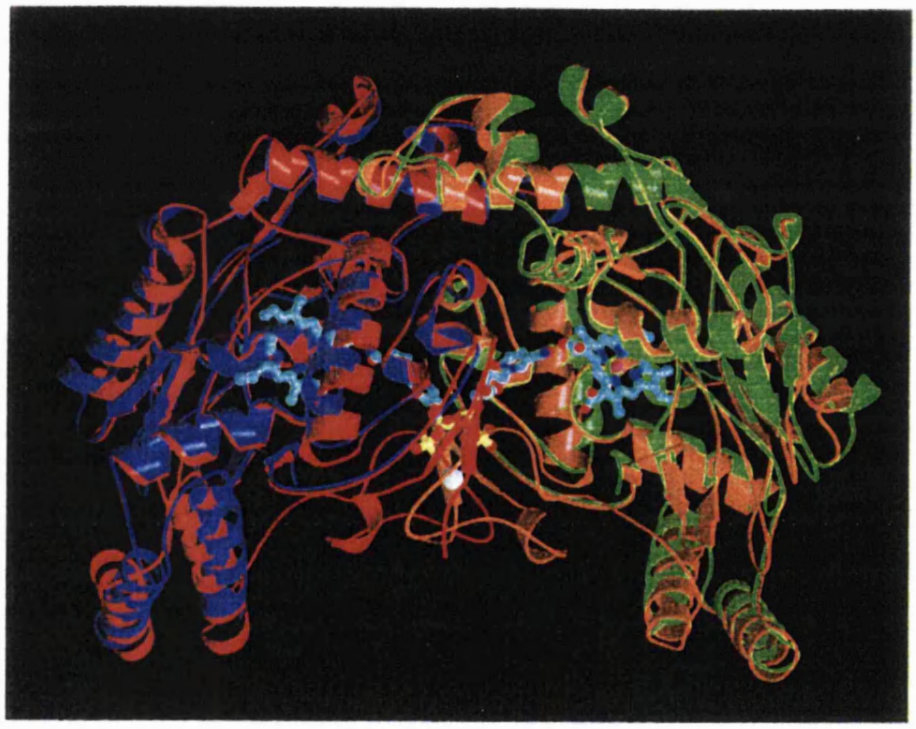

Figure I (upper right). C_α Trace of the SANOS dimer overlaid onto C_α Trace of the bovine eNOS dimer (-BH_4, +SEITU; 3NSE). The subunits are coloured blue and green, and, red and orange respectively. The hemes, and the interface $NADH_2$, from

SANOS are shown as ball and stick models, and, the heme iron is represented by a magenta sphere. The interface zinc in bovine eNOS is shown as a white sphere.

Appendix 5 Alignment of SANOS and human NOS isoforms

```
HeNOS  ------------------------------------------------------------
HnNOS  MEDHMFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
HiNOS  ------------------------------------------------------------
SANOS  ------------------------------------------------------------

HeNOS  ------------------------------------------------MGN------ 3
HnNOS  GDIILAVNGRPLVDLSYDSALEVLRGIASETHVVLILRGPEGFTTHLETTFTGDGTPKTI 120
HiNOS  ----------------------------------MACPWKFLFRVKS--YQGDLKE--  20
SANOS  ------------------------------------------------------------

HeNOS  ---------LKSVAQEPGPPCG----------LGLGLG-------------LGLCGKQGP 31
HnNOS  RVTQPLGPPTKAVDLSHQPPAGKEQPLAVDGASGPGNGPQHAYDDGQEAGSLPHANGLAP 180
HiNOS  ---------EKDINNNVEKTPG--------------------------AIPSPTTQDD 43
SANOS  ------------------------------------------------------------

HeNOS  ATPAPEPSR------------------------------------------------- 40
HnNOS  RPPGQDPAKKATRVSLQGRGENNELLKEIEPVLSLLTSGSRGVKGGAPAKAEMKDMGIQV 240
HiNOS  PKSHKHQNG---------------------------------------------- 52
SANOS  ------------------------------------------------------------

HeNOS  ------------------------------APASLLPPAPEHSPPSSPLTQ-------PP 63
HnNOS  DRDLDGKSHKPLPLGVENDRVFNDLWGKGNVPVVLNNPYSEKEQPPTSGKQSPTKNGSPS 300
HiNOS  ------------------------------FPQFLTGTAQNVPESLDKLHVT------PS 76
SANOS  ------------------------------------------------------------

HeNOS  EGPKFPRVKNWEVGSITYDTLSAQAQQDGPCTPRRCLGSLVFPRKLQGRPSPGPPAPEQL 123
HnNOS  KCPRFLKVKNWETEVVLTDTLHLKSTLETGCTEYICMGSIMHPSQHARRPE-DVRTKGQL 359
HiNOS  TRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIMNSKSLTRGPRDKPTPVEEL 136
SANOS  ------------------------------------------------------------ML 2
                                                                     *

HeNOS  LSQARDFINQYYSSIKRSGSQAHEQRLQEVEAEVAATGTYQLRESELVFGAKQAWRNAPR 183
HnNOS  FPLAKEFIDQYYSSIKRFGSKAHMERLEEVNKEIDTTSTYQLKDTELIYGAKHAWRNASR 419
HiNOS  LPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTLDELIFATKMAWRNAPR 196
SANOS  FKEAQAFIENMYKECH-YETQIINKRLHDIELEIKETGTYTHTEEELIYGAKMAWRNSNR 61
       :  *  **:: * . :    :    **. :  *:  *.**     **::.:* ****: *

HeNOS  CVGRIQWGKLQVFDARDCRSAQEMFTYICNHIKYATNRGNLRSAITVFPQRCPGRGDFRI 243
HnNOS  CVGRIQWSKLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSAITIFPQRTDGKHDFRV 479
HiNOS  CIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSAITVFPQRNDGKHDFRI 256
SANOS  CIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPYITIYAPK-DGP---KI 117
       *:**: *..*:*:***.      ::  *  *:  *** *:::. **::. :  *    ::

HeNOS  WNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDEPPELF 303
HnNOS  WNSQLIRYAGYKQPDGSTLGDPANVQFTEICIQQGWKPPRGRFDVLPLLLQANGNDPELF 539
HiNOS  WNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFDVLPLVLQAHGQDPEVF 316
SANOS  FNNQLIRYAGYDN-----CGDPAEKEVTRLANHLGWKGKGTNFDVLPLIYQLPNESVKFY 172
       :*.**:*****        ****  :.*.:. . **.    .******: *  .:  :.:
```

```
HeNOS  LLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWYMSTEIG 363
HnNOS  QIPPELVLEVPIRHPKFEWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWYMGTEIG 599
HiNOS  EIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGLEFPACPFNGWYMGTEIG 376
SANOS  EYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGGIVYPTAPFNGWYMVTEIG 232
         *..*: **.:.*     :  *.*:**.:* ::** *::**: :.:.**.**** ****

HeNOS  TRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINVAVLHSYQLAKVTIVDHHAA 423
HnNOS  VRDYCDNSRYNILEEVAKKMNLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTIVDHHSA 659
HiNOS  VRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAAVLHSFQKQNVTIMDHHTA 436
SANOS  VRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAVYHSFKKEGVSIVDHLTA 292
       .*:  *  ***:**.*.  : :.  .  *: **:* .*:* ** :*::   *:*:** :*

HeNOS  TASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQPDPWKGS 483
HnNOS  TESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPSFEYQPDPWNTH 719
HiNOS  SESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLNYVLSPFYYYQIEPWKTH 496
SANOS  AKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDNTVKDPNFFYK------- 345
       : .*  . .** :     ...* *:.**:* ::*. :*:   *    * : *:

HeNOS  AAKGTGITRKKT----FKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSYAQQLG 539
HnNOS  VWKGTNGTPTKRRAIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATETGKSQAYAKTLC 779
HiNOS  IWQDEKLRPRRR-EIRFTVLVKAVFFASVLMRKVMASRVRATVLFATETGKSEALARDLA 555
SANOS  ----------------------------------------------------KKESNAN--- 352
                                                             : :: *.

HeNOS  RLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMSGPYNS 599
HnNOS  EIFKHAFDAKVMSMEEYDIVHLEHETLVLVVTSTFGNGDPPENGEKFGCALMEMRHPNSV 839
HiNOS  ALFTYAFNTKVVCMEQYKANTLEEEQLLLVVTSTFGNGDCPSNGQTLKKSLFMMK----- 610
SANOS  ------------------------------------------------------------

HeNOS  SPRPEQHKSYKIRFNSISCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGSRAYPH 659
HnNOS  Q---EERKSYKVRFNSVSSYSDSQKSSGDGPDLRDNFESAGPLANVRFSVFGLGSRAYPH 896
HiNOS  --------------------------------ELG---------HTFRYAVFGLGSSMYPQ 630
SANOS  ------------------------------------------------------------

HeNOS  FCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGEDA--K 717
HnNOS  FCAFGHAVDTLLEELGGERILKMREGDELCGQEEAFRTWAKKVFKAACDVFCVGDDVNIE 956
HiNOS  FCAFAHDIDPKLSHLGASQLAPTGEGDELSGQEDAFRSWAVQTFRAACETFDVRSKHCIQ 690
SANOS  QCPFHH------------------------------------------------------ 358
        *.* :

HeNOS AAARDIFSPKRSWKRQRYRLSAQAEGLQLLPGLIHVHRRKMFQATIRSVENLQSSKSTRA 777
HnNOS KANNSLISNDRSWKRNKFRLTFVAEAPELTQGLSNVHKKRVSAARLLSRQNLQSPKSSRS 1016
HiNOS IPKR--YTSNATWEPEQYKLTQSPEPLDLNKALSSIHAKNVFAMRLKSLQNLQSEKSSRT 748
SANOS ------------------------------------------------------------

HeNOS TILVRLDTGGQEGLQYQPGDHIGVCPPNRPGLVEALLSRVEDPPAPTEPVAVEQLE-KGS 836
HnNOS TIFVRLHTNGSQELQYQPGDHLGVFPGNHEDLVNALIERLEDAPPVNQMVKVELLEERNT 1076
HiNOS TLLVQLTFEGSRGPSYLPGEHLGIFPGNQTALVQGILERVVDCSSPDQTVCLEVLDESGS 808
SANOS ------------------------------------------------------------
```

```
HeNOS PGGPPPGWVRDPRLPPCTLRQALTFFLDITSPPSPQLLRLLSTLAEEPREQQELEALSQD 896
HnNOS ALGVISNWTDELRLPPCTIFQAFKYYLDITTPPTPLQLQQFASLATSEKEKQRLLVLSKG 1136
HiNOS Y------WVKDKRLPPCSLRQALTYFLDITTPPTQLQLHKLARFATEETHRQRLEALCQ- 861
SANOS ------------------------------------------------------------


HeNOS PRRYEEWKWFRCPTLLEVLEQFPSVALPAPLLLTQLPLLQPRYYSVSSAPSTHPGEIHLT 956
HnNOS LQEYEEWKWGKNPTIVEVLEEFPSIQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLT 1196
HiNOS PSEYNDWKFSNNPTFLEVLEEFPSLRVPAAFLLSQLPILKPRYYSISSSQDHTPSEVHLT 921
SANOS ------------------------------------------------------------


HeNOS VAVLAYRTQDGLGPLHYGVCSTWLSQLKPGDPVPCFIRGAPSFRLPPDPSLPCILVGPGT 1016
HnNOS VAIVSYRTRDGEGPIHHGVCSSWLNRIQADELVPCFVRGAPSFHLPRNPQVPCILVGPGT 1256
HiNOS VAVVTYRTRDGQGPLHHGVCSTWINNLKPEDPVPCFVRSVSGFQLPEDPSQPCILIGPGT 981
SANOS ------------------------------------------------------------


HeNOS GIAPFRGFWQERLHDIESKGLQPTPMTLVFGCRCSQLDHLYRDEVQNAQQRGVFGRVLTA 1076
HnNOS GIAPFRSFWQQRQFDIQHKGMNPCPMVLVFGCRQSKIDHIYREETLQAKNKGVFRELYTA 1316
HiNOS GIAPFRSFWQQRLHDSQRRGLKGGRMTLVFGCRHPEEDHLYQEEMQEMVRKGVLFQVHTG 1041
SANOS ------------------------------------------------------------


HeNOS FSREPDNPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATNVLQTVQRILATEGD 1136
HnNOS YSREPDKPKKYVQDILQEQLAESVYRALKEQGGHIYVCGDVTMAADVLKAIQRIMTQQGK 1376
HiNOS YSRLPGKPKVYVQDILQKELADEVFSVLHGEQGHIYVCGDVRMARDVATTLKKLVAAKLN 1101
SANOS ------------------------------------------------------------


HeNOS MELDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERQLRGAVPWAFDPP 1196
HnNOS LSAEDAGVFISRMRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DTDEVFSS- 1434
HiNOS LSEEQVEDYFFQLKSQKRYHEDIFGAVFSYGVKKGNALEEPKGTRL-------------- 1147
SANOS ------------------------------------------------------------


HeNOS GSDTNSP 1203
HnNOS -------
HiNOS -------
SANOS -------
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Appendix 6 Alignment of the *yflM*-encoded protein and human NOS isoforms

```
HeNOS  ------------------------------------------------------------
HnNOS  MEDHMFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
HiNOS  ------------------------------------------------------------
yflM   ------------------------------------------------------------

HeNOS  ----------------------------------------------------MGN------ 3
HnNOS  GDIILAVNGRPLVDLSYDSALEVLRGIASETHVVLILRGPEGFTTHLETTFTGDGTPKTI 120
HiNOS  ----------------------------------MACPWKFLFRVKS-YQGDLKE--- 20
yflM   ------------------------------------------------------------

HeNOS  ---------LKSVAQEPGPPCG----------LGLGLG-------------LGLCGKQGP 31
HnNOS  RVTQPLGPPTKAVDLSHQPPAGKEQPLAVDGASGPGNGPQHAYDDGQEAGSLPHANGLAP 180
HiNOS  ---------EKDINNNVEKTPG--------------------------AIPSPTTQDD 43
yflM   ------------------------------------------------------------

HeNOS  ATPAPEPSR------------------------------------------------------ 40
HnNOS  RPPGQDPAKKATRVSLQGRGENNELLKEIEPVLSLLTSGSRGVKGGAPAKAEMKDMGIQV 240
HiNOS  PKSHKHQNG------------------------------------------------------ 52
yflM   ------------------------------------------------------------

HeNOS  ------------------------------APASLLPPAPEHSPPSSPLTQ-------PP 63
HnNOS  DRDLDGKSHKPLPLGVENDRVFNDLWGKGNVPVVLNNPYSEKEQPPTSGKQSPTKNGSPS 300
HiNOS  ------------------------------FPQFLTGTAQNVPESLDKLHVT------PS 76
yflM   ------------------------------------------------------------

HeNOS  EGPKFPRVKNWEVGSITYDTLSAQAQQDGPCTPRRCLGSLVFPRKLQGRPSPGPPAPEQL 123
HnNOS  KCPRFLKVKNWETEVVLTDTLHLKSTLETGCTEYICMGSIMHPSQHARRPE-DVRTKGQL 359
HiNOS  TRPQHVRIKNWGNGEIFHDTLHHKATSDISCKSKLCMGSIMNSKSLTRGPRDKPTPVEEL 136
yflM   ------------------------------------------------------------

HeNOS  LSQARDFINQYYSSIKRSGSQAHEQRLQEVEAEVAATGTYQLRESELVFGAKQAWRNAPR 183
HnNOS  FPLAKEFIDQYYSSIKRFGSKAHMERLEEVNKEIDTTSTYQLKDTELIYGAKHAWRNASR 419
HiNOS  LPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTLDELIFATKMAWRNAPR 196
yflM   ----------------------MKDRLADIKSEIDLTGSYVHTKEELEHGAKMAWRNSNR 38
                              **  :  *:  *.:*     ** ..:* ****: *

HeNOS  CVGRIQWGKLQVFDARDCRSAQEMFTYICNHIKYATNRGNLRSAITVFPQRCPGRGDFRI 243
HnNOS  CVGRIQWSKLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSAITIFPQRTDGKHDFRV 479
HiNOS  CIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSAITVFPQRNDGKHDFRI 256
yflM   CIGRLFWNSLNVIDRRDVRTKEEVRDALFHHIETATNNGKIRPTITIFPPEEKGEKQVEI 98
       *:**: *..*:*:* *.  :   :   : .*:  *** *::*.:**:** .  *. :..:

HeNOS  WNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDEP-PEL 302
HnNOS  WNSQLIRYAGYKQPDGSTLGDPANVQFTEICIQQGWKPPRGRFDVLPLLLQANGND-PEL 538
HiNOS  WNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFDVLPLVLQAHGQD-PEV 315
yflM   WNHQLIRYAGYE-SDGERIGDPASCSLTAACEELGWRGERTDFDLLPLIFRMKGDEQPVW 157
       ** **:*****.  **   ****. .:*  * : **      **:***:::  .:  *
```

```
HeNOS   FLLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWYMSTEI 362
HnNOS   FQIPPELVLEVPIRHPKFEWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWYMGTEI 598
HiNOS   FEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGLEFPACPFNGWYMGTEI 375
yflM    YELPRSLVIEVPITHPDIEAFSDLELKWYGVPIISDMKLEVGGIHYNAAPFNGWYMGTEI 217
        : :* .**:**.: **  * *  * *:**.:* :::* **:**:.: *.**.****.***

HeNOS   GTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINVAVLHSYQLAKVTIVDHHA 422
HnNOS   GVRDYCDNSRYNILEEVAKKMNLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTIVDHHS 658
HiNOS   GVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAAVLHSFQKQNVTIMDHHT 435
yflM    GARNLADEKRYDKLKKVASVIGIAADYNTDLWKDQALVELNKAVLHSYKKQGVSIVDHHT 277
        *.*: .*  **: *:.*.  :.:     :.****:* .*:* ***:*::   *:*:***:


HeNOS   ATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQPDPWKG 482
HnNOS   ATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPSFEYQPDPWNT 718
HiNOS   ASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLNYVLSPFYYYQIEPWKT 495
yflM    AASQFKRFEEQEEEAGRKLTGDWTWLIPPISPAATHIFHRSYDNSIVKPNYFYQDKPYE- 336
        *: .* :. ::* ..    ..** *::**:* : * :**:.  *  :.* : ** .*::


HeNOS   SAAKGTGITRKKT----FKEVANAVKISASLMGTVMAKRVKATILYGSETGRAQSYAQQL 538
HnNOS   HVWKGTNGTPTKRRAIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATETGKSQAYAKTL 778
HiNOS   HIWQDEKLRPRRR-EIRFTVLVKAVFFASVLMRKVMASRVRATVLFATETGKSEALARDL 554
yflM    ------------------------------------------------------------


HeNOS   GRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAAALMEMSGPYN 598
HnNOS   CEIFKHAFDAKVMSMEEYDIVHLEHETLVLVVTSTFGNGDPPENGEKFGCALMEMRHPNS 838
HiNOS   AALFTYAFNTKVVCMEQYKANTLEEEQLLLVVTSTFGNGDCPSNGQTLKKSLFMMK---- 610
yflM    ------------------------------------------------------------


HeNOS   SSPRPEQHKSYKIRFNSISCSDPLVSSWRRKRKESSNTDSAGALGTLRFCVFGLGSRAYP 658
HnNOS   VQ---EERKSYKVRFNSVSSYSDSQKSSGDGPDLRDNFESAGPLANVRFSVFGLGSRAYP 895
HiNOS   --------------------------------ELG---------HTFRYAVFGLGSSMYP 629
yflM    ------------------------------------------------------------


HeNOS   HFCAFARAVDTRLEELGGERLLQLGQGDELCGQEEAFRGWAQAAFQAACETFCVGEDA-- 716
HnNOS   HFCAFGHAVDTLLEELGGERILKMREGDELCGQEEAFRTWAKKVFKAACDVFCVGDDVNI 955
HiNOS   QFCAFAHDIDPKLSHLGASQLAPTGEGDELSGQEDAFRSWAVQTFRAAACETFDVRSKHCI 689
yflM    ------------------------------------------------------------


HeNOS KAAARDIFSPKRSWKRQRYRLSAQAEGLQLLPGLIHVHRRKMFQATIRSVENLQSSKSTR 776
HnNOS EKANNSLISNDRSWKRNKFRLTFVAEAPELTQGLSNVHKKRVSAARLLSRQNLQSPKSSR 1015
HiNOS QIPKR--YTSNATWEPEQYKLTQSPEPLDLNKALSSIHAKNVFAMRLKSLQNLQSEKSSR 747
yflM  ------------------------------------------------------------


HeNOS ATILVRLDTGGQEGLQYQPGDHIGVCPPNRPGLVEALLSRVEDPPAPTEPVAVEQLE-KG 835
HnNOS STIFVRLHTNGSQELQYQPGDHLGVFPGNHEDLVNALIERLEDAPPVNQMVKVELLEERN 1075
HiNOS TTLLVQLTFEGSRGPSYLPGEHLGIFPGNQTALVQGILERVVDCSSPDQTVCLEVLDESG 807
yflM  ------------------------------------------------------------


HeNOS SPGGPPPGWVRDPRLPPCTLRQALTFFLDITSPPSPQLLRLLSTLAEEPREQQELEALSQ 895
HnNOS TALGVISNWTDELRLPPCTIFQAFKYYLDITTPPTPLQLQQFASLATSEKEKQRLLVLSK 1135
HiNOS SY------WVKDKRLPPCSLRQALTYFLDITTPPTQLQLHKLARFATEETHRQRLEALCQ 861
yflM  ------------------------------------------------------------
```

```
HeNOS DPRRYEEWKWFRCPTLLEVLEQFPSVALPAPLLLTQLPLLQPRYYSVSSAPSTHPGEIHL 955
HnNOS GLQEYEEWKWGKNPTIVEVLEEFPSIQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHL 1195
HiNOS -PSEYNDWKFSNNPTFLEVLEEFPSLRVPAAFLLSQLPILKPRYYSISSSQDHTPSEVHL 920
yflM  ------------------------------------------------------------

HeNOS TVAVLAYRTQDGLGPLHYGVCSTWLSQLKPGDPVPCFIRGAPSFRLPPDPSLPCILVGPG 1015
HnNOS TVAIVSYRTRDGEGPIHHGVCSSWLNRIQADELVPCFVRGAPSFHLPRNPQVPCILVGPG 1255
HiNOS TVAVVTYRTRDGQGPLHHGVCSTWINNLKPEDPVPCFVRSVSGFQLPEDPSQPCILIGPG 980
yflM  ------------------------------------------------------------

HeNOS TGIAPFRGFWQERLHDIESKGLQPTPMTLVFGCRCSQLDHLYRDEVQNAQQRGVFGRVLT 1075
HnNOS TGIAPFRSFWQQRQFDIQHKGMNPCPMVLVFGCRQSKIDHIYREETLQAKNKGVFRELYT 1315
HiNOS TGIAPFRSFWQQRLHDSQRRGLKGGRMTLVFGCRHPEEDHLYQEEMQEMVRKGVLFQVHT 1040
yflM  ------------------------------------------------------------

HeNOS AFSREPDNPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATNVLQTVQRILATEG 1135
HnNOS AYSREPDKPKKYVQDILQEQLAESVYRALKEQGGHIYVCGDVTMAADVLKAIQRIMTQQG 1375
HiNOS GYSRLPGKPKVYVQDILQKELADEVFSVLHGEQGHIYVCGDVRMARDVATTLKKLVAAKL 1100
yflM  ------------------------------------------------------------

HeNOS DMELDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERQLRGAVPWAFDP 1195
HnNOS KLSAEDAGVFISRMRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DTDEVFSS 1434
HiNOS NLSEEQVEDYFFQLKSQKRYHEDIFGAVFSYGVKKGNALEEPKGTRL------------- 1147
yflM  ------------------------------------------------------------

HeNOS PGSDTNSP 1203
HnNOS --------
HiNOS --------
yflM  --------
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Appendix 7 Multialignment of NOS and SANOS.

```
Drosophila  ------------------------------------------------------------
mosquito    ------------------------------------------------------------
Rhop        ------------------------------------------------------------
hornworm    ------------------------------------------------------------
Lyms        ------------------------------------------------------------
HiNOS       ------------------------------------------------------------
RatINOS     ------------------------------------------------------------
mouseiNOS   ------------------------------------------------------------
GpigiNOS    ------------------------------------------------------------
DogiNOS     ------------------------------------------------------------
ChickeniNOS ------------------------------------------------------------
CarpiNOS    ------------------------------------------------------------
BoseNOS     ------------------------------------------------------------
PigeNOS     ------------------------------------------------------------
DogeNOS     ------------------------------------------------------------
HeNOS       ------------------------------------------------------------
GpigeNOS    ------------------------------------------------------------
mouseeNOS   ------------------------------------------------------------
mousenNOS   MEEHTFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
RatnNOS     MEENTFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
HnNOS       MEDHMFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
RabbitnNOS  MEEHVFGVQQIQPNVISVRLFKRKVGGLGFLVKERVSKPPVIISDLIRGGAAEQSGLIQA 60
SANOS       ------------------------------------------------------------
```

```
Drosophila  --------------------MSQHFTSIFENLRFVTIKRATN---------AQQQQQQQQ 31
mosquito    --------------------MADTTTVVVERREVAEGRESSK---------ANHIGEERR 31
Rhop        --------------------MVHAECVWWLGIRILFVPPVSL---------EMHSVNVNN 31
hornworm    --------------------MSNIFKICCMKDPSGSVKDTLG---------KMEHVNGHF 31
Lyms        ------------------------------------------------------------
HiNOS       --------------------MACPWKFLFRVKSYQGDLKEE----------KDINNNVEK 30
RatINOS     --------------------MACPWKFLFKVKSYQGDLKEE----------KDINNNVEK 30
mouseiNOS   --------------------MACPWKFLFKVKSYQSDLTEE----------KDINNNVKK 30
GpigiNOS    --------------------MACPWNFLWKLKSSRYDLTEE----------KDINNNVGK 30
DogiNOS     --------------------MACPWKFLFRAKFHQYGMKEE----------KDINNNVEK 30
ChickeniNOS --------------------MLCPWQFAFKPHAVKNQSSEE----------KDINNNVEK 30
CarpiNOS    --------------------MGNQATKANKNATPHQITPNT----------QCENNNVIL 30
BoseNOS     --------------------MGNLKSVGQEP-------GPP----------CGLGLGLGL 23
PigeNOS     --------------------MGNLKSVGQEP-------GPP----------CGLGLGLGL 23
DogeNOS     --------------------MGNLKSVGQEP-------GPP----------CGLGLGLGL 23
HeNOS       --------------------MGNLKSVAQEP-------GPP----------CGLGLGLGL 23
GpigeNOS    --------------------MGNLKSVGQEP-------GPP----------CGLGLGLGL 23
mouseeNOS   --------------------MGNLKSVGQEP-------GPP----------CGLGLGLGL 23
mousenNOS   GDIILAVNDRPLVDLSYDSALEVLRGIASETHVVLILRGPEGFTTHLETTFTGDGTPKTI 120
RatnNOS     GDIILAVNDRPLVDLSYDSALEVLRGIASETHVVLILRGPEGFTTHLETTFTGDGTPKTI 120
HnNOS       GDIILAVNGRPLVDLSYDSALEVLRGIASETHVVLILRGPEGFTTHLETTFTGDGTPKTI 120
RabbitnNOS  GDIILAVNGRPLVDLSYDSALEVLRGVASETHVVLILRGPEGFTTNLETTFTGDGTPKTI 120
SANOS       ------------------------------------------------------------
```

```
Drosophila   QQQLQQQQQQLQQQKAQTQQQNSRKIKTQATPTLNGNGLLSGNPNGGGGDSSPSHEVDHP  91
mosquito     GYDVSRKR----------------------CSISVHG-----GGTEGGGGNMRTNYR----  61
Rhop         MSIQQQQQ-----------------------HQPQ-------------------------  43
hornworm     VPSKCPFSGE--------------------SDFKVDN-----------QKTVKPNLR----  57
Lyms         ------------------------------------------------------------
HiNOS        TPGAIPSPTTQDDPKSH-------------------------------------------  47
RatINOS      TPGAIPSPTTQDDPKSH-------------------------------------------  47
mouseiNOS    TPCAVLSPTIQDDPKS--------------------------------------------  46
GpigiNOS     AS-HLYSPEIQDDPKYCSP-----------------------------------------  48
DogiNOS      PPGATPSPSTQDDLKNH-------------------------------------------  47
ChickeniNOS  DVKVHSF--VKDDAKLHSL---------------------------------------  47
CarpiNOS     KKTTPNTQCENNNVILQP------------------------------------------  48
BoseNOS      GLCGKQGPASPAPEPSRAP-----------------------------------------  42
PigeNOS      GLCGKQGPATPAPEPSRAP-----------------------------------------  42
DogeNOS      GLCGKQGPASPTSEPSRAP-----------------------------------------  42
HeNOS        GLCGKQGPATPAPEPSRAP-----------------------------------------  42
GpigeNOS     GLCGKQGPASPAPVSASEP-----------------------------------------  42
mouseeNOS    GLCGKQGPASPAPEPSQAP-----------------------------------------  42
mousenNOS    RVTQPLGTPTKAVDLSRQP-SASKDQPLAVDRVPGPSNGPQHAQGRGQGAGSVSQANGVA 179
RatnNOS      RVTQPLGPPTKAVDLSHQP-SASKDQSLAVDRVTGLGNGPQHAQGHGQGAGSVSQANGVA 179
HnNOS        RVTQPLGPPTKAVDLSHQP-PAGKEQPLAVDGASGPGNGPQHAYDDGQEAGSLPHANGLA 179
RabbitnNOS   RVTQPLGAPTKAVDLSHQPPSAGKEQPRPVDGAAGPGSWPQPTQGHGQEAGSPSRANGLA 180
SANOS        ------------------------------------------------------------
```

```
Drosophila   GGAQGAQAAGGLPSLSGTPLRHHKRASISTASPPIRERRGTNTSIVVELDGSGSGSGSGG 151
mosquito     -------------ELSPASLRIHRKS-----SHDIRNTLLGPDGEVLHLHDP---SGKGG 100
Rhop         ------------------------------------------------------------
hornworm     ------------------------------------------------------------
Lyms         ------------------------------------------------------------
HiNOS        ------------------------------------------------------------
RatINOS      ------------------------------------------------------------
mouseiNOS    ------------------------------------------------------------
GpigiNOS     ------------------------------------------------------------
DogiNOS      ------------------------------------------------------------
ChickeniNOS  ------------------------------------------------------------
CarpiNOS     ------------------------------------------------------------
BoseNOS      ------------------------------------------------------------
PigeNOS      ------------------------------------------------------------
DogeNOS      ------------------------------------------------------------
HeNOS        ------------------------------------------------------------
GpigeNOS     ------------------------------------------------------------
mouseeNOS    ------------------------------------------------------------
mousenNOS    -----IDPTMKNTKANLQDSGEQDELLKEIEPVLSILTGGGKAVNRGGPAKAEMKDTGIQ 234
RatnNOS      -----IDPTMKSTKANLQDIGEHDELLKEIEPVLSILNSGSKATNRGGPAKAEMKDTGIQ 234
HnNOS        PRPPGQDPAKKATRVSLQGRGENNELLKEIEPVLSLLTSGSRGVKGGAPAKAEMKDMGIQ 239
RabbitnNOS   PRTSSQDPAKKSGWAGLQGSGDKNELLKEIEPVLTLLAGGSKAVDGGGPAKAETRDTGVQ 240
SANOS        ------------------------------------------------------------
```

```
Drosophila  GGVGVGQGAGCPPSGSCTASGKSSRELSPSPKNQQQPRKMSQDYRSRAGSFMHLDDEGRS 211
mosquito    ----------------------------------------------------DGMGKM 106
Rhop        ------------------------------------------------------------
hornworm    ------------------------------------------------------------
Lyms        ------------------------------------------------------------
HiNOS       --------------------------------KHQNGFPQFLTGTAQNVPESLDKLHVTP 75
RatiNOS     --------------------------------KHQNGFPQFLTGTAQNVPESLDKLHVTP 75
mouseiNOS   --------------------------------HQNGSPQLLTGTAQNVPESLDKLHVT- 72
GpigiNOS    -------------------------------GKHQNGSSQSLTGTAKKVPESQSKPHKPS 77
DogiNOS     --------------------------------KHHNDSPQPLTETVQKLPESLDKLHATP 75
ChickeniNOS -------------------------------SKKQMKMSPIIT-SAEKHPQNGIKASNQI 75
CarpiNOS    --------------------------------ITPNMCENNNVILQQITPN-MKWKNKV 74
BoseNOS     ---------------------------------APATPHAPDHSPAPN-SPTLTR---P 64
PigeNOS     ---------------------------------APATPHAPEHSPAPN-SPTLTR---P 64
DogeNOS     ---------------------------------ALAPPPSPPPAPDHS-SPPLTR---P 64
HeNOS       ---------------------------------ASLLPPAPEHSPP---SSPLTQ---P 62
GpigeNOS    ---------------------------------TRAPSSPPLPLPAPEHSPPLTR---P 65
mouseeNOS   ---------------------------------APPSPTR----AAPDHSPPLTR---P 61
mousenNOS   VDRDLDGKLHKAPPLGGENDRVFNDLWGKGNVPVVLNNPYSENEQSPASGKQSPTKNGSP 294
RatnNOS     VDRDLDGKSHKAPPLGGDNDRVFNDLWGKDNVPVILNNPYSEKEQSPTSGKQSPTKNGSP 294
HnNOS       VDRDLDGKSHKPLPLGVENDRVFNDLWGKGNVPVVLNNPYSEKEQPPTSGKQSPTKNGSP 299
RabbitnNOS  VDRDFDAKSHKPLPLGVENDRVFSDLWGKGSAPVVLNNPYSEKEQPPASGKQSPTKNGSP 300
SANOS       ------------------------------------------------------------
```

```
Drosophila  LLMRKPMRLKNIEGRPEVYDTLHCKGREIL----SCSKATCTSSIMNIGN----AAVEAR 263
mosquito    PAVVKPIKLKSIVTKAESYDTMHGKASDVM----SCSREVCMGSVMTPHV----IGTETR 158
Rhop        QQLLKPIRLANVSTQAQSLDTLHYKCQQEG----PCMEQACLASVIYAGVN---LKPRVR 96
hornworm    IKVPQPIRLKNHLVNEENFDTLHSRIDEVTSFNTKCTEKVCQTSLMDIPN----RGDTPR 113
Lyms        ----------------------------------------MGSLSQQAHG---PPDAPR 16
HiNOS       STRPQHVRIKNWGNGEIFHDTLHHKATSDI----SCKSKLCMGSIMNSKSLTRGPRDKPT 131
RatINOS     STRPQHVRIKNWGNGEIFHDTLHHKATSDI----SCKSKLCMGSIMNSKSLTRGPRDKPT 131
mouseiNOS   STRPQYVRIKNWGSGEILHDTLHHKATSDF----TCKSKSCLGSIMNPKSLTRGPRDKPT 128
GpigiNOS    PTCSQHMKIKNWGNGMILQDTLHTKAKTNF----TCKPKSCLGSVMNPRSMTRGPRDTPI 133
DogiNOS     LSRPQHVRIKNWGNGRSFQDTLHHKAMGVL----ACTSKLCMGSIMNTKSLTRGPSDKPT 131
ChickeniNOS SRCPRHVKVRNMENGSSLLDTLHLTAKEVI----NCRTRACQGALMTPKGLVRSTRDGPV 131
CarpiNOS    NRCPFSKQLKNYQDGLFHQDTLHSRAVKSQ----ICMSNVCEGSVMTPKAMTRCPSSTMP 130
BoseNOS     PEGPKFPRVKNWELGSITYDTLCAQSQQDG----PCTPRCCLGSLVLPRKLQTRPSPGPP 120
PigeNOS     PEGPKFPRVKNWEVGSITYDTLCAQSQQDG----PCTPRRCLGSLVLPRKLQSRPSPGPP 120
DogeNOS     PDGPKFPRVKNWEVGSITYDTLSAQSQQDG----PCTPRRCLGSLVFPRKLQSRPSQNPA 120
HeNOS       PEGPKFPRVKNWEVGSITYDTLSAQAQQDG----PCTPRRCLGSLVFPRKLQGRPSPGPP 118
GpigeNOS    PEGPKFPRVKNWEVGSIAYDTLSAQAQQDG----PCTPRRCLGSLVFPRKLQGRPSQSPL 121
mouseeNOS   PDGPRFPRVKNWEVGSITYDTLSAQAQQDG----PCTSRRCLGSLVFPRKLQSRPTQGPS 117
mousenNOS   SRCPRFLKVKNWETDVVLTDTLHLKSTLET----GCTEQICMGSIMLPSHHIRKS-EDVR 349
RatnNOS     SRCPRFLKVKNWETDVVLTDTLHLKSTLET----GCTEHICMGSIMLPSQHTRKP-EDVR 349
HnNOS       SKCPRFLKVKNWETEVVLTDTLHLKSTLET----GCTEYICMGSIMHPSQHARRP-EDVR 354
RabbitnNOS  SKCPRFLKVKNWETDVVLTDTLHLKSTLET----GCTEHICMGSIMFPSQHTRRP-EDIR 355
SANOS       ------------------------------------------------------------
```

```
Drosophila   KSDLILEHAKDFLEQYFTSIKRTSCTAHETRWKQVRQSIETTGHYQLTETELIYGAKLAW 323
mosquito     KPEIVQQHAKDFLDQYYSSIRRLKSPAHDSRWQQVQKEVEATGSYHLTETELIYGAKLAW 218
Rhop         PKEELLAHAKDFLDQYFASIRRLQSPAHEARWAQVEKEVAATGTYELTETELVYGAKLAW 156
hornworm     TAEEVFQDAQTFLRQYFASIRRENSEAHTARLEEVKRELKDKGSYQLKTSELVFGAKLAW 173
Lyms         SKEELLIHAKDFINQYFTSFQMNKTRAHFHRLGEINDLIEKSGTYDLTMAELTFGAKHAW 76
HiNOS        PVEELLPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTLDELIFATKMAW 191
RatINOS      PVEELLPQAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTLDELIFATKMAW 191
mouseiNOS    PLEELLPHAIEFINQYYGSFKEAKIEEHLARLEAVTKEIETTGTYQLTLDELIFATKMAW 188
GpigiNOS     PPDELLPQAIEFVNQYYDSFKEAKIEEYLARVETVTKEIETTGTYQLTGDELIFATKLAW 193
DogiNOS      PTEELLPQAIEFVNQYYGSFKEAKIEEHLARVEAVTKDIETTGTYQLTGDELIFATKQAW 191
ChickeniNOS  PPAELLPQAVDFVKQYYSSFKELKIEEHLARLETVTKEIETTGTYHLTKDELIFAAKQAW 191
CarpiNOS     GSDDILTQAVDFINQYYKSIKNSKIEEHLSRLEEVTKEIEATGSYRLTTKELEFGAKQAW 190
BoseNOS      PAEQLLSQARDFINQYYSSIKRSGSQAHEERLQEVEAEVASTGTYHLRESELVFGAKQAW 180
PigeNOS      PAEQLLSQARDFINQYYSSIKRSGSQAHEERLQEVEAEVATTGTYHLGESELVFGAKQAW 180
DogeNOS      PPEQLLSQARDFISQYYSSIKRSGSQAHEQRLQEVEAEVAATGTYQLRESELVFGAKQAW 180
HeNOS        APEQLLSQARDFINQYYSSIKRSGSQAHEQRLQEVEAEVAATGTYQLRESELVFGAKQAW 178
GpigeNOS     PQEQLLGQARDFINQYYSSIKRSGSQAHELRLQEVEAEVVATGTYQLRESELVFGAKQAW 181
mouseeNOS    PTEQLLGQARDFINQYYNSIKRSGSQAHEQRLQEVEAEVAATGTYQLRESELVFGAKQAW 177
mousenNOS    TKDQLFPLAKEFLDQYYSSIKRFGSKAHMDRLEEVNKEIESTSTYQLKDTELIYGAKHAW 409
RatnNOS      TKDQLFPLAKEFLDQYYSSIKRFGSKAHMDRLEEVNKEIESTSTYQLKDTELIYGAKHAW 409
HnNOS        TKGQLFPLAKEFIDQYYSSIKRFGSKAHMERLEEVNKEIDTTSTYQLKDTELIYGAKHAW 414
RabbitnNOS   TKEQLFPLAKEFIDQYYSSIKRFGSKAHMERLEEVNKEIESTSTYQLKDTELIYGAKHAW 415
SANOS        ---MLFKEAQAFIENMYKECH-YETQIINKRLHDIELEIKETGTYTHTEEELIYGAKMAW 56
                 :   *  *: : : . :         *   :   :  .. *     ** :.:* **
```

```
Drosophila   RNSSRCIGRIQWSKLQVFDCRYVTTTSGMFEAICNHIKYATNKGNLRSAITIFPQRTDAK 383
mosquito     RNSSRCIGRIQWSKLQVFDCRYVTTTSGMFEAICNHIKYATNKGNLRSAITIFPQRTDGK 278
Rhop         RNAPRCIGRIQWAKLQVFDCRQVTTTSGMFEALCNHIKYSTNKGNIRSAITIFPHRTDGK 216
hornworm     RNATRCIGRIQWKKLQTFDCREVTTASGMFEALCNHIKYATNKGNIRSAITIFPQRTDGK 233
Lyms         RNAPGCIGRSQWSKLQVFDAREIGTPREMFEALCSHIRYATNEGKIRSTITIFPQRKEGR 136
HiNOS        RNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSAITVFPQRNDGK 251
RatINOS      RNAPRCIGRIQWSNLQVFDARSCSTASEMFQHICRHILYATNSGNIRSAITVFPQRSDGK 251
mouseiNOS    RNAPRCIGRIQWSNLQVFDARNCSTAQEMFQHICRHILYATNNGNIRSAITVFPQRSDGK 248
GpigiNOS     RNAPRCIGRIQWSNLQVFDARSCHTAQEMFEHICRHVRYSTNNGNIRSAITVFPQRTDGK 253
DogiNOS      RNAPRCIGRIQWSNLQVFDARSCSTAKEMFEHICRHLRYASNNGNIRSAITVFPQRTDGK 251
ChickeniNOS  RNAPRCIGRIQWSNLQVFDARDCKTAKEMFEYICRHIQYATNNGNIRSAITIFPQRTDGK 251
CarpiNOS     RNAPRCIGRIQWANLQLFDARKCRTAEDMFQMLCDHIQFATNGGNVRSAITVFPQRTDGQ 250
BoseNOS      RNAPRCVGRIQWGKLQVFDARDCSSAQEMFTYICNHIKYATNRGNLRSAITVFPQRAPGR 240
PigeNOS      RNAPRCVGRIQWGKLQVFDARDCSSAQEMFTYICNHIKYATNRGNLRSAITVFPQRTPGR 240
DogeNOS      RNAPRCVGRIQWGKLQVFDARDCSSAQEMFTYICNHIKYATNRGNLRSAITVFPQRASGR 240
HeNOS        RNAPRCVGRIQWGKLQVFDARDCRSAQEMFTYICNHIKYATNRGNLRSAITVFPQRCPGR 238
GpigeNOS     RNAPRCVGRIQWGKLQVFDARDCRSAQEMFTYICNHIKYATNRGNLRSAITVFPQRFPGR 241
mouseeNOS    RNAPRCVGRIQWGKLQVFDARDCRTAQEMFTYICNHIKYATNRGNLRSAITVFPQRCPGR 237
mousenNOS    RNASRCVGRIQWSKLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSAITIFPQRTDGK 469
RatnNOS      RNASRCVGRIQWSKLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSAITIFPQRTDGK 469
HnNOS        RNASRCVGRIQWSKLQVFDARDCTTAHGMFNYICNHVKYATNKGNLRSAITIFPQRTDGK 474
RabbitnNOS   RNASRCVGRIQWSKLQVFDARDCTTAHGMFNYICNHIKYATNKGNLRSAITIFPQRTDGK 475
SANOS        RNSNRCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPYITIYAPK-DGP 115
             **:  *:**  * .*: :*.*       ::  :  *:  ::* *:::. **::. :  .
```

```
Drosophila   HDYRIWNNQLISYAGYKQADGKIIGDPMNVEFTEVCTKLGWKSKGSEWDILPLVVSANGH 443
mosquito     HDYRIWNNQIISYAGYKNADGKIIGDPANVEFTDFCVKLGWKSKRTEWDILPLVVSANGH 338
Rhop         HDFRIWNKQLISYAGHKSKDGTVIGDPACVEFTEICIKLGWKGKGTMFDVLPLVLSANGE 276
hornworm     HDYRIWNPQLIGYAGYQEPDGSILGDPARVEFTEVCLKLGWKPARTAWDILPLVLSADGK 293
Lyms         PDFRVWNTQLISYAGYKLGDGKVIGDPANVEFTEMCVEMGWKPKHGMFDLLPLVLSAAEN 196
HiNOS        HDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFDVLPLVLQAHGQ 311
RatINOS      HDFRIWNSQLIRYAGYQMPDGTIRGDPATLEFTQLCIDLGWKPRYGRFDVLPLVLQAHGQ 311
mouseiNOS    HDFRLWNSQLIRYAGYQMPDGTIRGDAATLEFTQLCIDLGWKPRYGRFDVLPLVLQADGQ 308
GpigiNOS     HDFRVWNAQLIRYAGYQMPDGTIQGDPANLEFTQLCIDLGWKPRYGRFDVLPLILQADGR 313
DogiNOS      HDFRVWNAQLIRYAGYQMPDGTILGDPASVEFTQLCIDLGWKPKYGRFDVVPLVLQADGQ 311
ChickeniNOS  HDFRVWNSQLIRYAGYQMPDGSVIGDPASVEFTKLCIELGWKPKYGRFDVVPLILQANGQ 311
CarpiNOS     HDFRVWNSQLIRYAGYKMTDGTIIGDPASVDFTEICIELGWTPRYGQFDVLPLVLQATEE 310
BoseNOS      GDFRIWNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 300
PigeNOS      GDFRIWNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 300
DogeNOS      GDFRIWNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 300
HeNOS        GDFRIWNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 298
GpigeNOS     GDFRIWNSQLIRYAGYRQQDGSVRGDPANVEITELCVQHGWTPGNGRFDVLPLLLQAPDE 301
mouseeNOS    GDFRIWNSQLIRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 297
mousenNOS    HDFRVWNSQLIRYAGYKQPDGSTLGDPANVEFTEICIQQGWKPPRGRFDVLPLLLQANGN 529
RatnNOS      HDFRVWNSQLIRYAGYKQPDGSTLGDPANVQFTEICIQQGWKAPRGRFDVLPLLLQANGN 529
HnNOS        HDFRVWNSQLIRYAGYKQPDGSTLGDPANVQFTEICIQQGWKPPRGRFDVLPLLLQANGN 534
RabbitnNOS   HDFRVWNSQLIRYAGYKQPDGSTLGDPANVQFTEICIQQGWKPPRSRFDVLPLLLQANGN 535
SANOS        ---KIFNNQLIRYAGYDN-----CGDPAEKEVTRLANHLGWKGKGTNFDVLPLIYQLPNE 167
                :::* *:: ***:        **.   :.* .. . **.     :*::**: .   .

Drosophila   DP-DYFDYPPELILEVPLTHPKFEWFSDLGLRWYALPAVSSMLFDVGGIQFTATTFSGWY 502
mosquito     DP-DYFDYPPELILEVPLSHPQFKWFAELNLRWYAVPMVSSMLFDCGGIQFTATAFSGWY 397
Rhop         DP-DYFDLPPELVFEVPLSHPKYKWFSELGLKWFALPAVSGMMFDCGGLQFTAAPFNGWY 335
hornworm     DP-EYFEIPREIVMEVQIVHPKYDWFKELGLQWYALPAVSNMRLDCGGLEFTATAFNGWY 352
Lyms         SP-EYFELPTELVLEVTLKHPEYPWFAEMGLKWYALPTDSGMLLDCGGLEFPSCPFNGWF 255
HiNOS        DP-EVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGLEFPACPFNGWY 370
RatINOS      DP-EVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGLEFPACPFNGWY 370
mouseiNOS    DP-EVFEIPPDLVLEVTMEHPKYEWFQELGLKWYALPAVANMLLEVGGLEFPACPFNGWY 367
GpigiNOS     DP-ELFEIPPDLVLEVPMEHPKYEWFQDLGLKWYALPAVANMLLEVGGLEFPACPFNGWY 372
DogiNOS      DP-EFFEIPPDLVLEVPMEHPKYEWFRELELKWYALPAVANMLLEVGGLEFPGCPFNGWY 370
ChickeniNOS  DP-EIFEYPPEIILEVPMEHPKYEWFKELDLKWYALPAVANMLLEVGGLEFTACPFNGWY 370
CarpiNOS     DPSVFLKFPQHLILEVPMKHQQYKWFKDLNLRWFALPAVSNMLLEIGGLEFPACPFNGWY 370
BoseNOS      AP-ELFVLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFSAAPFSGWY 359
PigeNOS      PP-ELFALPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWY 359
DogeNOS      PP-ELFALPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWY 359
HeNOS        PP-ELFLLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWY 357
GpigeNOS     PP-ELFTLPPELVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAVPFSGWY 360
mouseeNOS    SP-ELFTLPPEMVLEVPLEHPTLEWFAALGLRWYALPAVSNMLLEIGGLEFPAAPFSGWY 356
mousenNOS    DP-ELFQIPPELVLEVPIRHPKFDWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWY 588
RatnNOS      DP-ELFQIPPELVLEVPIRHPKFDWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWY 588
HnNOS        DP-ELFQIPPELVLEVPIRHPKFEWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWY 593
RabbitnNOS   DP-ELFQIPPELVLEVPIRHPKFEWFKDLGLKWYGLPAVSNMLLEIGGLEFSACPFSGWY 594
SANOS        SV-KFYEYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGGIVYPTAPFNGWY 226
                    *  :: ** : *     :  : *:*:.:*  :.* :. **: :.  .*.**:
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Drosophila   MSTEIGSRNLCDTNRRNMLETVALKMQLDTRTPTSLWKDKAVVEMNIAVLHSYQSRNVTI 562
mosquito     MSTEIGCRNLCDANRRNLLEPIAIKMGLDTRNPTSLWKDKALVEINIAVLHSYQSRNITI 457
Rhop         MNSEIGSRNLGDTNRYNMLEKIAQKMELDTRTPVTLWKDLAMVEANVAVLHSFQLHNVTI 395
hornworm     MGTEIGCRNFCDSNRLNVVENVARQMGLDTNSFVSLWKDKALVEVNIAVLHSYLRDNVSI 412
Lyms         MGTMIGSRNLCDPHRYNMLEPIGLKMGLNTETASSLWKDRVLIEVNVAVLYSFESANVTI 315
HiNOS        MGTEIGVRDLCDTQRYNILEEVGRRMGLGTHTLASLWKDRAVTEINAAVLHSFQKQNVTI 430
RatINOS      MGTEIGVRDLCDTQRYNILEEVGRRMGLETHTLASLWKDRAVTEINAAVLHSFQKQNVTI 430
mouseiNOS    MGTEIGVRDFCDTQRYNILEEVGRRMGLETHTLASLWKDRAVTEINVAVLHSFQKQNVTI 427
GpigiNOS     MGTEIGVRDFCDAQRYNILEEVGRRMGLETHTLASLWKDRAVTEINVAVLHSFQKQNVTI 432
DogiNOS      MGTEIGVRDFCDVQRYNILEEVGSKMGLETHKLASLWKDRAVIEINVAVLHSFQKQNVTI 430
ChickeniNOS  MGTEIGVRDFCDVQRYNILKEVGRRMGLESNKLASLWKDRAVVEINVAVLHSFQKQNVTI 430
CarpiNOS     MGTEIGVRDFCDTKRYNVLERVGRQMGLETQKLPSLWKDQALVAINVAVMHSFQKNKVTI 430
BoseNOS      MSTEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINLAVLHSFQLAKVTI 419
PigeNOS      MSTEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINLAVLHSYQLAKVTI 419
DogeNOS      MSTEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINLAVLHSYQLAKVTI 419
HeNOS        MSTEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINVAVLHSYQLAKVTI 417
GpigeNOS     MSSEIGMRNFCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINVAVLHSYQLAKVTI 420
mouseeNOS    MSSEIGMRDLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINVAVLHSYQLAKVTI 416
mousenNOS    MGTEIGVRDYCDNSRYNILEEVAKKMDLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTI 648
RatnNOS      MGTEIGVRDYCDNSRYNILEEVAKKMDLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTI 648
HnNOS        MGTEIGVRDYCDNSRYNILEEVAKKMNLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTI 653
RabbitnNOS   MGTEIGVRDYCDNSRYNILEEVAKKMNLDMRKTSSLWKDQALVEINIAVLYSFQSDKVTI 654
SANOS        MVTEIGVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAVYHSFKKEGVSI 286
             * : ** *:  *  * *::: :.  : :   .  :: ** .    * ** :*:    ::*


Drosophila   VDHHTASESFMKHFENESKLRNGCPADWIWIVPPLSGSITPVFHQEMALYYLKPSFEYQD 622
mosquito     VDHHTASESFMKHFENETKLRNGCPADWIWIVPPMSASVTPVFHQEMAVYYLRPSFEYQE 517
Rhop         VDHHSAAESFMKHLENEQRLRGGCPADWVWIVPPISGSATPVFFQEMANYFLYPGYIYQE 455
hornworm     VDHHSASEQFLKHLDNENKSRGGCPADWIWIVPPMSSSLTSVFHQEMALYYIRPSYDYQE 472
Lyms         VNHHDASTDFISHMDKEIKLRGGCPSDWVRMVPPMSGSTLEVFHQEMLLYNLHPAFVRQD 375
HiNOS        MDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLNYVLSPFYYYQI 490
RatINOS      MDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLNYVLSPFYYYQI 490
mouseiNOS    MDHHTASESFMKHMQNEYRARGGCPADWIWLVPPVSGSITPVFHQEMLNYVLSPFYYYQI 487
GpigiNOS     MDHHSAAESFMKHMQNEYRARGGCPADWIWLVPPISGSITPVFHQEMLNYILSPFYYYQV 492
DogiNOS      MDHHSAAESFMKYMQSEYRSRGGCPADWIWLVPPISGSITPVFHQEMLNYVLSPFYYYQV 490
ChickeniNOS  MDHHSAAESFMKYMQNEYRVRGGCPADWVWIVPPMSGSITPVFHQEMLNYVLTPFFYYQV 490
CarpiNOS     TDHHTAPESFMQHMEMEVRLRGGCPADWVWLVPPMSGSLTPVYHQEMLNYILSPFFYYQP 490
BoseNOS      VDHHAATVSFMKHLDNEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYILSPAFRYQP 479
PigeNOS      VDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYVLSPAFRYQP 479
DogeNOS      VDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYVLSPAFRYQT 479
HeNOS        VDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQP 477
GpigeNOS     VDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQP 480
mouseeNOS    VDHHAATASFMKHLENEQKARGGCPADWAWIVPPISGSLTPVFHQEMVNYFLSPAFRYQP 476
mousenNOS    VDHHSATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPSFEYQP 708
RatnNOS      VDHHSATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPSFEYQP 708
HnNOS        VDHHSATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPSFEYQP 713
RabbitnNOS   VDHHSATESFIKHMENEYRCRGGCPADWVWIVPPMSGSITPVFHQEMLNYRLTPCFEYQP 714
SANOS        VDHLTAAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDNTVKDPNFFYKK 346
              :*  *. .*  .   * :     ...*  :.**:* :    :.:        * :  :
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Drosophila   P-AWRTHVWKKGRGESKGKK-PRRKFNFKQIARAVKFTSKLFGRALSKRIKATVLYATET 680
mosquito     S-AMKTHIWKKGRDSAKNKK-PRRKFNFKQIARAVKFTSKLFGRALSRRIKATVLYATET 575
Rhop         D-AWKCHEWKEIDVKHGLKK-EKRKFHFKQIARAVKFTSKLFGSALSKRIKATILFATET 513
hornworm     P-AWKTHQWTK---SDGTKA-VTRKYHFKQIARAVKFTSKLFGRALSKRIKATILYATET 527
Lyms         VKPWKKHVWKSDQSVPINSCNPKRKLGFKALARAVEFSASLMSKALSSRVKCSIFYATET 435
HiNOS        E-PWKTHIWQDEKLRPRRR-----EIRFTVLVKAVFFASVLMRKVMASRVRATVLFATET 544
RatINOS      E-PWKTHIWQDEKLRPRRR-----EIRFTVLVKAVFFASVLMRKVMASRVRATVLFATET 544
mouseiNOS    E-PWKTHIWQNEKLRPRRR-----EIRFRVLVKVVFFASMLMRKVMASRVRATVLFATET 541
GpigiNOS     E-AWKTHVWQDETRRPKRR-----EIPFRVLAKATLFASLLMRKMMASRVRATILFATET 546
DogiNOS      E-AWKTHLWLDEKRRPHRK-----KIQLKVLVKAVLFASMLMRKTMASRVRVTILFATET 544
ChickeniNOS  D-AWKTHIWHDETRRPKKR-----EIKLSILAKAVLLASLLLQKTMAARPKVTVIYATET 544
CarpiNOS     D-PWLTHKWKVKKRNARRH-----TISFKGLIRAVLFSQTLIKSALTKRVHCTVLYATET 544
BoseNOS      D-PWKGSATKGAGITRKKT--------FKEVANAVKISASLMGTLMAKRVKATILYASET 530
PigeNOS      D-PWKGSAAKGTGIARKKT--------FKEVANAVKISASLMATVMPKRVKASILYASET 530
DogeNOS      D-PWKGSASKGAGVTRKKT--------FKEVANAVKISASLMGTVMAKRVKATILYGSET 530
HeNOS        D-PWKGSAAKGTGITRKKT--------FKEVANAVKISASLMGTVMAKRVKATILYGSET 528
GpigeNOS     D-PWKGSGTKGTGITRKKT--------FKEVANAVKISASLMGTVMAKRVKATILYGSET 531
mouseeNOS    D-PWKGSAAKGAGITRKKT--------FKEVANAVKISASLMGTVMAKRVKATILYGSET 527
mousenNOS    D-PWNTHVWKGTNGTPTKRR----AIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATET 763
RatnNOS      D-PWNTHVWKGTNGTPTKRR----AIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATET 763
HnNOS        D-PWNTHVWKGTNGTPTKRR----AIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATET 768
RabbitnNOS   D-PWNTHVWKGTNGTPTKRR----AIGFKKLAEAVKFSAKLMGQAMAKRVKATILYATET 769
SANOS        K--------------------------ESNANQCPFHH---------------------- 358
                                          .   :
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Drosophila   GKSEQYAKQLCELLGHAFNAQIYCMSDYDISSIEHEALLIVVASTFGNGDPPENGELFSQ 740
mosquito     GRSEQYARQLVELLGHAFNAQIYCMSDYDISSIEHEALLLVVASTFGNGDPPENGELFAQ 635
Rhop         GKSEMYARKLGDIFSHAFHSQVLSMEDYDMSKIEHEALLLVVASTFGNGDPPENGQGFAQ 573
hornworm     GKSEQYAKELGTIFGHAFNAQVHCMSEYDMFSIEHETLLLIVTSTFGNGEPPANGVDFTE 587
Lyms         GRSERFARRLSEIFKPVFHSRVVCMDDYAVETLEHESLVMVITSTFGNGEPPENGKQFAQ 495
HiNOS        GKSEALARDLAALFTYAFNTKVVCMEQYKANTLEEEQLLLVVTSTFGNGDCPSNGQTLKK 604
RatINOS      GKSEALARDLAALFSYAFNTKVVCMEQYKANTLEEEQLLLVVTSTFGNGDCPSNGQTLKK 604
mouseiNOS    GKSEALARDLATLFSYAFNTKVVCMDQYKASTLEEEQLLLVVTSTFGNGDCPSNGQTLKK 601
GpigiNOS     GKSEALAQDLGALFSCAFNPKVLCMDQYQLSSLEEEKLLLVVTSTFGNGDCPGNGETLKK 606
DogiNOS      GKSETLARDLGALFSCAFHPKVLCMDEYKLSHLEEEQLLLVVTSTFGNGDSPGNGEKLKK 604
ChickeniNOS  GKSETLANSLCSLFSCAFNTKILCMDEYNISDLEKETLLLVVTSTFGNGDSPNNGKTLKN 604
CarpiNOS     GKSHTFAKKLNTMMNCAFKSQVVSMEDYNFSELEKESFLIVVTSTFGNGDCPGNGESFKK 604
BoseNOS      GRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAA 590
PigeNOS      VRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAA 590
DogeNOS      GRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAA 590
HeNOS        GRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAA 588
GpigeNOS     GRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHETLVLVVTSTFGNGDPPENGESFAA 591
mouseeNOS    GRAQSYAQQLGRLFRKAFDPRVLCMDEYDVVSLEHEALVLVVTSTFGNGDPPENGESFAA 587
mousenNOS    GKSQAYAKTLCEIFKHAFDAKAMSMEEYDIVHLEHEALVLVVTSTFGNGDPPENGEKFGC 823
RatnNOS      GKSQAYAKTLCEIFKHAFDAKAMSMEEYDIVHLEHEALVLVVTSTFGNGDPPENGEKFGC 823
HnNOS        GKSQAYAKTLCEIFKHAFDAKVMSMEEYDIVHLEHETLVLVVTSTFGNGDPPENGEKFGC 828
RabbitnNOS   GKSQAYAKTLCEIFKHAFDAKVMSMEEYDIVHLEHETLVLVVTSTFGNGDPPENGEKFRC 829
SANOS        ------------------------------------------------------------
```

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Drosophila   ELYAMRVQESSEHGLQDSS-------IGSSKSFMKASSRQEFMKLPLQQVKRIDRWDSLR 793
mosquito     DLYAMKLHESGHHQAHSELT-----IAASSKSFIKANSRSDLGKFGPMGGRKIDRLDSLR 690
Rhop         SLYTIKMDENGLPNGHTNN------TLASSASFIKANSQTDRQAS-------LERCDSFR 620
hornworm     HLFQMLYNESKNQGDQTGDLGSGNFKTQTPKSLMRTNS---MMTPSFEYKRQLTRLESNK 644
Lyms         SLLDMKRKYDCDLG------------FLESCSSISTCIKSSILTEGPLAADVIGDRQS-- 541
HiNOS        SLFMMK---------------------------------------------------- 610
RatINOS      SLFMMK---------------------------------------------------- 610
mouseiNOS    SLFMLR---------------------------------------------------- 607
GpigiNOS     SLFVLK---------------------------------------------------- 612
DogiNOS      SLFMLK---------------------------------------------------- 610
ChickeniNOS  SLLTLK---------------------------------------------------- 610
CarpiNOS     QLLSLN---------------------------------------------------- 610
BoseNOS      ALMEMSGPYN----------------------SSPRPEQHKSYKIRFNSVSCSDPLVSSWR 629
PigeNOS      ALMEMSGPYN----------------------GSPRPEQHRSYKIRFNSVSCSDPLVSSWR 629
DogeNOS      ALMEMSGSYN----------------------SSPRPEQHKSYKIRFNSVSCSDPLVSSWR 629
HeNOS        ALMEMSGPYN----------------------SSPRPEQHKSYKIRFNSISCSDPLVSSWR 627
GpigeNOS     ALMEMSGPYN----------------------SSPRPEQHKSYKIRFNSVSCSDPLVTSWR 630
mouseeNOS    ALMEMSGPYN----------------------SSPRPEQHKSYKIRFNSVSCSDPLVSSWR 626
mousenNOS    ALMEMRHPNS----------------------VQ---EERKSYKVRFNSVSSYSDSRKSSG 859
RatnNOS      ALMEMRHPNS----------------------VQ---EERKSYKVRFNSVSSYSDSRKSSG 859
HnNOS        ALMEMRHPNS----------------------VQ---EERKSYKVRFNSVSSYSDSQKSSG 864
RabbitnNOS   ALMEMRHPNS----------------------LQ---EERKSYKVRFNSVSSYSDSRKSSG 865
SANOS        ------------------------------------------------------------
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Drosophila   GSTSDTFTEETFGPLSNVRFAVFALGSSAYPNFCAFGQYVDNILGELGGERLLRVAYGDE 853
mosquito     GSTTDTLSEETFGPLSNVRFAVFALGSSAYPNFCAFGKYIDNILGELGGERLMKMATGDE 750
Rhop         GSTGD---ADVFGPLSNVRFAVFALGSSAYPNFCAFGSYVDNLLGELGGERLVKLTTGDE 677
hornworm     SSIAGTSTAEQIGPLSNVCFAVFALGSSAYPKFCNFGKTVDKVLGDLGGERILELACGDE 704
Lyms         -------LAMGTGPLCNVRFAVFGLGSKAYPYYAAYGKYIYLMLQELGAERLVNYCAGDA 594
HiNOS        ------------ELGHTFRYAVFGLGSSMYPQFCAFAHDIDPKLSHLGASQLAPTGEGDE 658
RatINOS      ------------ELGHTFRYAVFGLGSSMYPQFCAFAHDIDQKLSHLGASQLAPTGEGDE 658
mouseiNOS    ------------ELNHTFRYAVFGLGSSMYPQFCAFAHDIDQKLSHLGASQLAPTGEGDE 655
GpigiNOS     ------------KLTNTFRYAVFGLGSSMYPRFCAFAHDIDIKLSQLGASQLTPVGEGDE 660
DogiNOS      ------------ELTNNFRYAVFGLRSNMYPQFCAFAHDIDHKLSHLGASQLTPGGEGDE 658
ChickeniNOS  ------------LLRKNIRYAVFGLGSTMYPEFCAFAHAIDQKLSQLGALQLTPVGEGDE 658
CarpiNOS     ------------NLRNQVRYCVFGLGSRMYPHFCAFAHAVDDRFAALGAIRVSATGEGDE 658
BoseNOS      RKRKESSNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDE 689
PigeNOS      RKRKESSNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDE 689
DogeNOS      RKRKESRNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDE 689
HeNOS        RKRKESSNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDE 687
GpigeNOS     RKRKESSNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLVQGDE 690
mouseeNOS    RKRKESSNTDSAGALGTLRFCVFGLGSRAYPHFCAFARAVDTRLEELGGERLLQLGQGDE 686
mousenNOS    DGPDLRDNFESTGPLANVRFSVFGLGSRAYPHFCAFGHAVDTLLEELGGERILKMREGDE 919
RatnNOS      DGPDLRDNFESTGPLANVRFSVFGLGSRAYPHFCAFGHAVDTLLEELGGERILKMREGDE 919
HnNOS        DGPDLRDNFESAGPLANVRFSVFGLGSRAYPHFCAFGHAVDTLLEELGGERILKMREGDE 924
RabbitnNOS   DGPDVRDHFESAGPLANVRFSVFGLGSRAYPHFCAFGHAVDTLLEELGGERILKMREGDE 925
SANOS        ------------------------------------------------------------
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Drosophila   MCGQEQSFRKWAPEVFKLACETFCLDPEESLSDASLA--LQNDSLTVNTVRLVPSANKGS 911
mosquito     ICGQEQAFRKWAPEVFKIACETFCLDPEETLSDAAFA--LQS-ELSENTVRYAPVAEYES 807
Rhop         MCGQAQACNKWAPEVFSVACDTFCLDSDETFLEATQM--LHSEAVTASTVRFVESAT-QD 734
hornworm     LYGQEQQFRTWSSNIFHVACETFCLDENDMVKDAKKA--LGTVPLTEETVRFGKPTTNPT 762
Lyms         LYGQEQSFRAWSEEVFKASCEAFCLDNRN---DAPGP--QTKGDCSKVRIVPVENCQEPD 649
HiNOS        LSGQEDAFRSWAVQTFRAACETFDVRSKHCIQIPKR--YTSNATWEPEQYKLTQSPEPLD 716
RatINOS      LSGQEDAFRSWAVQTFRAACETFDVRSKHCIQIPKR--YTSNATWEPEQYKLTQSPEPLD 716
mouseiNOS    LSGQEDAFRSWAVQTFRAACETFDVRSKHHIQIPKR--FTSNATWEPQQYRLIQSPEPLD 713
GpigiNOS     LSGQEDAFCTWAVQTFQAACAAFDVRGRHHITIPKR--YTSSVTWEPYHYRLVQDSQPLD 718
DogiNOS      LNGKEEAFRCWAVQTFKAACDTSDVRGKHCIQIPRL--YTSNVTWDPHHYRLLQDSQPLD 716
ChickeniNOS  LNGQEEAFRTWAVTAFKTACDIFDIRGKNSIQLPEI--YTSDDSWNPKKHRIVYDSQTMD 716
CarpiNOS     LNGQEEAFSAWACTVFKDACKEFNIQ----CELPGK--EGMADSWDPQRHRVQNDSCTVD 712
BoseNOS      LCGQEEAFRGWAKAAFQASCETFCVGEEA--KAAAQDIFSPKRSWKRQRYRLSTQAEGLQ 747
PigeNOS      LCGQEEAFRGWAQAAFQASCETFCVGEDA--KAAARDIFSPKRSWKRQRYRLSAQVEGLQ 747
DogeNOS      LCGQEEAFGGWAQAAFQASWETFCVGEDA--KAAARDIFSPKRTWKRQRYRLSAQAEGLQ 747
HeNOS        LCGQEEAFRGWAQAAFQAACETFCVGEDA--KAAARDIFSPKRSWKRQRYRLSAQAEGLQ 745
GpigeNOS     LCGQEEAFRGWAQAAFQAACETFCVGEDA--KAAAKDIFSPKCSWKRQRYRLSTQAQGLQ 748
mouseeNOS    LCGQEEAFRGWAQAAFQAACETFCVGEDA--KAAARDIFSPKRSWKRQRYRLSTQAESLQ 744
mousenNOS    LCGQEEAFRTWAKKVFKAACDVFCVGDDVNIEKANNSLISNDRSWKRNKFRLTYVAEAPE 979
RatnNOS      LCGQEEAFRTWAKKVFKAACDVFCVGDDVNIEKPNNSLISNDRSWKRNKFRLTYVAEAPD 979
HnNOS        LCGQEEAFRTWAKKVFKAACDVFCVGDDVNIEKANNSLISNDRSWKRNKFRLTFVAEAPE 984
RabbitnNOS   LCGQEEAFRTWAKKVFKAACDVFCVGDDVNIEKANNSLISNDRSWKRNKFRLTYVAEAPG 985
SANOS        ------------------------------------------------------------
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Drosophila LDSSLSKYHNKKVHCCKAKAKPHNLTRLSEG-AKTTMLLEICAPG----LEYEPGDHVGI 966
mosquito   LDRALSKFHNKKSMECSVKRNPINLHCEMNGTERSTILVEIMAEG----IDYEPGDHVGI 863
Rhop       LCKALSHLHNKKVWKCPLLGK-RNLH--GKGSTRATLLLEIERNEN---ISYQPGDHVGV 788
hornworm   LKTALEAGFRKQLIVCKVKEN---KHLGDYSVDRATIFVDMEPQSE---FKYDPGDHVGV 816
Lyms       LCQVLRNIHGKEVMPLILAER---IQLQAKDSDQQTILIKLDAHNATD-LKYAPGDHVAI 705
HiNOS      LNKALSSIHAKNVFAMRLKSL---QNLQSEKSSRTTLLVQLTFEGSRG-PSYLPGEHLGI 772
RatINOS    LNKALSSIHAKNVFTMRLKSL---QNLQSEKSSRTTLLVQLTFEGSRG-PSYLPGEHLGI 772
mouseiNOS  LNRALSSIHAKNVFTMRLKSQ---QNLQSEKSSRTTLLVQLTFEGSRG-PSYLPGEHLGI 769
GpigiNOS   LNKALSRMHATDVFTMRLKSQ---KNLQSPKSSRTTLLMELSCDDSRS-LAYLPGEHLGV 774
DogiNOS    LNKALSKMHAKNVFTLRLKSQ---RNLQSPISNRTTLQVELSCEDSQE-LSYLPGEHLGV 772
ChickeniNOSLTKALSDIHGKNVIPMKLKFR---QNLQSLKSSRVTILVKLSCETNQE-VHYLPGEHIGI 772
CarpiNOS   RITALSALHSKAVVPMKLKRR---QNLQSPTSSRATILVELGMDGNTEPLNIVPGDHVGI 769
BoseNOS    LLPGLIHVHRRKMFQATVLSV---ENLQSSKSTRATILVRLDTAGQEG-LQYQPGDHIGI 803
PigeNOS    LLPGLVHVHRRKMFQATVLSV---ENLQSSKSTRATILVRLDTEGQEG-LQYQPGDHIGI 803
DogeNOS    LLPGLIHVHRRKMVQATVLAV---ENLQSSKSTRATILVRLDTGSQEA-LQYQPGDHIGI 803
HeNOS      LLPGLIHVHRRKMFQATIRSV---ENLQSSKSTRATILVRLDTGGQEG-LQYQPGDHIGV 801
GpigeNOS   LLPGLIHVHRRKMFQATILSV---ENLQSSKSTRATILVRLDTGGQEG-LQYQPGDHIGI 804
mouseeNOS  LLPGLTHVHRRKMFQATILSV---ENLQSSKSTRATILVRLDTGGQEG-LQYQPGDHIGV 800
mousenNOS  LTQGLSNVHKKRVSAARLLSR---QNLQSPKSSRSTIFVRLHTNGNQE-LQYQPGDHLGV 1035
RatnNOS    LTQGLSNVHKKRVSAARLLSR---QNLQSPKFSRSTIFVRLHTNGNQE-LQYQPGDHLGV 1035
HnNOS      LTQGLSNVHKKRVSAARLLSR---QNLQSPKSSRSTIFVRLHTNGSQE-LQYQPGDHLGV 1040
RabbitnNOS LTQGLSSVHKKRVSAARLLSR---QNLQSPKSSRSTIFVRLHTNGSQE-LQYQPGDHLGV 1041
SANOS      ------------------------------------------------------------
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Drosophila FPANRTELVDGLLNRLVGVDNPDEVLQLQLLKEKQTSNGIFKCWEPHDKIPPDTLRNLLA 1026
mosquito   FPANRKEIVDGIIERLTGVNDPDEMLQLQVLKEKQTQNGVYKSWEPHERLPVCTLRTLLT 923
Rhop       LACNRKELVEGIISHLESAIDPDKSVQLQILKENTTPDGIVRNWIPHDRLPTCSLRTMLT 848
hornworm   MACNRKEIVDAVLSRTKDDDNYDKQVQLQVMKETLTPTGAVKTWERHERIPAVTVREIFT 876
Lyms       FPANSPEIVDAILVRLDTSKGPSP--DQVVKTEISTQLGTNDTWRSH--LPICTSRTAFS 761
HiNOS      FPGNQTALVQGILERVVDCSSPDQTVCLEVLD--ESGS----YWVKDKRLPPCSLRQALT 826
RatINOS    FPGNQTALVQGILERVVDCSSPDQTVCLEVLD--ESGS----YWVKDKRLPPCSLRQALT 826
mouseiNOS  FPGNQTALVQGILERVVDCPTPHQTVCLEVLD--ESGS----YWVKDKRLPPCSLSQALT 823
GpigiNOS   FPCNQPALVQGILECVVDNPGPHHTVCLEVLD--DSGS----YWAKDKRLPPCSLSQALT 828
DogiNOS    FPGNQLALVQGILERVVYSPAPLQPVHLETLS--ERGS----YWVRNNRLPPCSLSQALT 826
ChickeniNOSSPGNQPELVHGLIARVKDAPPADQTIRLETCT--EGG-----YWASEKKIPACTLSQALT 825
CarpiNOS   FPGNSPELVAGILKHLANAPPINQSLRLEFLSACPDGE----RWQRVERIPPCSLAQALT 825
BoseNOS    CPPNRPGLVEALLSRVEDPPPPTESVAVEQLE-KGSPGGPPPSWVRDPRLPPCTLRQALT 862
PigeNOS    CPPNRTGLVEALLSRVEDPTPPTESVGVEQLE-KGSPGGPPPSWVRDPRLPPYTLRQALT 862
DogeNOS    CPPNRPGLVEALLSRVEDPPPPGEPVAVEQLE-KGSPGGPPPSWVRDPRLPPCTLRQALT 862
HeNOS      CPPNRPGLVEALLSRVEDPPAPTEPVAVEQLE-KGSPGGPPPGWVRDPRLPPCTLRQALT 860
GpigeNOS   CPPNRPGLVEALLSRVEDPPPPAESVAVEQLE-KGSPGGPPPGWVRDPRLPPCTLRQALT 863
mouseeNOS  CPPNRPGLVEALLSRVEDPPPSTEPVAVEQLE-KGSPGGPPPGWVRDPRLPPCTLRQALT 859
mousenNOS  FPGNHEDLVNALIERLEDAPPANHVVKVEMLEERNTALGVISNWKDESRLPPCTIFQAFK 1095
RatnNOS    FPGNHEDLVNALIERLEDAPPANHVVKVEMLEERNTALGVISNWKDESRLPPCTIFQAFK 1095
HnNOS      FPGNHEDLVNALIERLEDAPPVNQMVKVELLEERNTALGVISNWTDELRLPPCTIFQAFK 1100
RabbitnNOS FPGNHEDLVNALIERLEDAPPANQMVKVELLEERNTALGVISNWKDEPRLPPCTVFQAFK 1101
SANOS      ------------------------------------------------------------
```

```
Drosophila RFFDLTTPPSRQLLTLLAGFCEDTADKERLELLVNDSSAYEDWRHWRLPHLLDVLEEFPS 1086
mosquito   RFLDITTPPTRQLLTYLASCCGDKADEERLLMLANESSVYEDWRYWKLPHLLEVLEEFPS 983
Rhop       RFLDITTPPSPNLLQFFASCATNSEDQEKLTELATDSAAYEDWRYWKYPNLLEVLEEFPS 908
hornworm   RFLDITTPPSTTVLKYLANSCTDQQDAEKLLELATDSNKYDDWRHFHYPNLAEVLAQFPS 936
Lyms       FLLDVTTPPSQEILQVLATQASSDMDKHKLEQLASNSEAYEKWRLDLSPNILEILDEFPS 821
HiNOS      YFLDITTPPTQLQLHKLARFATEETHRQRLEALCQ-PSEYNDWKFSNNPTFLEVLEEFPS 885
RatINOS    YFLDITTPPTQLQLHKLARFATEETHRQRLEALCQ-PSEYNDWKFSNNPTFLEVLEEFPS 885
mouseiNOS  YFLDITTPPTQLQLHKLARFATDETDRQRLEALCQ-PSEYNDWKFSNNPTFLEVLEEFPS 882
GpigiNOS   YFLDITTPPTQLQLQKLARLATEQAERLRLESLSQ-PSEYNKWKFTNSPTFLEVLEEFPS 887
DogiNOS    YFLDITTPPTHLLLRKLAQLAHQYAERHRLEILCH-PSEYNKWKLTNSPTFLEVLEEFPS 885
ChickeniNOSYLLDITTPPTQQLLKKLSQLVTAEGDKQRLEVLCHSTEEYNKWKFYNRPNILEVLEEFPS 885
CarpiNOS   YYLDVTTPPSQSLLRKLSKMAKQEDHRQRLLALATDFQVYATWKEFYKPTSLEVLEEFSS 885
BoseNOS    FFLDITSPPSPRLLRLLSTLAEEPSEQQELETLSQDPRRYEEWKWFRCPTLLEVLEQFPS 922
PigeNOS    FFLDITSPPSPRLLRVLSTLAEEPSEQQELETLSQDPRRYEEWKWFRCPTLLEVLEQFPS 922
DogeNOS    FFLDITSPPSPQLLRLLSTLAEESSEQQELESLSQDPRRYEEWKWFRCPTLLEVLEQFPS 922
HeNOS      FFLDITSPPSPQLLRLLSTLAEEPREQQELEALSQDPRRYEEWKWFRCPTLLEVLEQFPS 920
GpigeNOS   FFLDITSPPSPRLLRLLSTLAEEPSEQQELETLSQDPRRYEEWKWFRCPTLLEVLEQFPS 923
mouseeNOS  YFLDITSPPSPRLLRLLSTLAEESSEQQELEALSQDPRRYEEWKWFSCPTLLEVLEQFPS 919
mousenNOS  YYLDITTPPTPLQLQQFASLATNEKEKQRLLVLSKGLQEYEEWKWGKNPTMVEVLEEFPS 1155
RatnNOS    YYLDITTPPTPLQLQQFASLATNEKEKQRLLVLSKGLQEYEEWKWGKNPTMVEVLEEFPS 1155
HnNOS      YYLDITTPPTPLQLQQFASLATSEKEKQRLLVLSKGLQEYEEWKWGKNPTIVEVLEEFPS 1160
RabbitnNOS YYLDITTPPTPLQLQQFASLASNEKEKQRLLVLSKGLQEYEEWKWGKNPTIVEVLEEFPS 1161
SANOS      ------------------------------------------------------------
```

```
Drosophila CRPPAPLLLAQLTPLQPRFYSISSSPRRVSDEIHLTVAIVKYRCEDGQGDERYGVCSNYL 1146
mosquito   CRPPAAVFVAQLNALQPRFYSISSSPRKYSNEIHLTVAIVTYRAEDGEGAEHYGVCSNYL 1043
Rhop       VRVLPALLIAQLTPLQPRFYSISSAPSLYANQIHLTVAVVQYCTQDGKGPIHYGVASNYL 968
hornworm   CKPQASLLAALLPPLQPRFYSISSSPVAHPERIHVTVAIVVYNTQNGKGPTHYGVCSTYL 996
Lyms       LKIPPSLLLTQLPLLQPRYYSISSSQQKNPNEVHATIAVVRFKTQDGDGPVHEGVCSSWL 881
HiNOS      LRVPAAFLLSQLPILKPRYYSISSSQDHTPSEVHLTVAVVTYRTRDGQGPLHHGVCSTWI 945
RatINOS    LRVPAAFLLSQLPILKPRYYSISSSQDHTPSEVHLTVAVVTYRTRDGQGPLHHGVCSTWI 945
mouseiNOS  LHVPAAFLLSQLPILKPRYYSISSSQDHTPSEVHLTVAVVTYRTRDGQGPLHHGVCSTWI 942
GpigiNOS   LRVPAAFLLSQLPILKPRYYSISSSLDHTPAEVHLTVAVVTYRTRDGRGPLHHGVCSTWF 947
DogiNOS    LRVSAGFLLSQLPILKPRYYSISSSRDCTPMEVHLTVAVLVYPTRDGQGPLHHGVCSTWL 945
ChickeniNOSAEVSTAFLLTQLPLLKPRYYSVSSSCDMTPREIHLTVAVVNYRTRDGQGPLHHGVCSTWL 945
CarpiNOS   LELSADFLLSQLPLLKPRLYSISSSPDLHPQELHLTVAVVSYCTQEGKGPLHFGLCSTWL 945
BoseNOS    VALPAPLLLTQLPLLQPRYYSVSSAPNAHPGEVHLTVAVLAYRTQDGLGPLHYGVCSTWL 982
PigeNOS    VALPTPLLLTQLALLQPRYYSVSSAPSTYPGEIHPTVAVLAYRTQDGLGPLHYGVCSTWL 982
DogeNOS    VALPAPLLLTQLPLLQPRYYSVSSAPSAHPGEIHLTVAVLAYRTQDGLGPLHYGVCSTWL 982
HeNOS      VALPAPLLLTQLPLLQPRYYSVSSAPSTHPGEIHLTVAVLAYRTQDGLGPLHYGVCSTWL 980
GpigeNOS   IALPAPLLLTQLPLLQPRYYSVSSAPSAHPGEIHLTVAELAYRTQDGLGPLHYGVCSTWL 983
mouseeNOS  VALPAPLILTQLPLLQPRYYSVSSAPSAHPGEIHLTIAVLAYRTQDGLGPLHYGVCSTWM 979
mousenNOS  IQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLTVAIVSYHTRDGEGPVHHGVCSSWL 1215
RatnNOS    IQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLTVAIVSYHTRDGEGPVHHGVCSSWL 1215
HnNOS      IQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLTVAIVSYRTRDGEGPIHHGVCSSWL 1220
RabbitnNOS IQMPATLLLTQLSLLQPRYYSISSSPDMYPDEVHLTVAIVSYHTRDGEGPIHHGVCSSWL 1221
SANOS      ------------------------------------------------------------
```

```
Drosophila SGLRADDELFMFVRSALGFHLPSDRSRPIILIGPGTGIAPFRSFWQEFQVLRDLDPTA-- 1204
mosquito   ANLQSDDKIYLFVRSAPSFHMSKDRTKPVILIGPGTGIAPFRSFWQEWDHIKTEMVDC-- 1101
Rhop       YDVTIGDSIYLFTRSAPNFHLPKSDTAPIIMVGPGTGIAPFRGFWQHRLAQRSLNGPG-- 1026
hornworm   QSLKPDDEVFVFIRRAPSFHMPKDVSAPLILVGPGSGVAPFRGFWHHRRHQMKNLVPNNK 1056
Lyms       NRSPIGTVVPCFLRSAPHFHLPEDPSLPIIMIGPGSGIAPFRSFWQQRLGEIENTMPSCE 941
HiNOS      NNLKPEDPVPCFVRSVSGFQLPEDPSQPCILIGPGTGIAPFRSFWQQRLHDSQRRGLK-- 1003
RatINOS    NNLKPEDPVPCFVRSVSGFQLPEDPSQPCILIGPGTGIAPFRSFWQQRLHDSQRRGLK-- 1003
mouseiNOS  RNLKPQDPVPCFVRSVSGFQLPEDPSQPCILIGPGTGIAPFRSFWQQRLHDSQHKGLK-- 1000
GpigiNOS   SGLKPQDPVPCLVRSVNSFQLPKDPSQPCILIGPGTGIAPFRSFWQQRLHNLKHTGLQ-- 1005
DogiNOS    SNLKPQDPVPCFVRSAGNFKLPEDPSRPCILIGPGTGIAPFRSFWQQRLHDIKHKGLR-- 1003
ChickeniNOSNKIALNETVPCFVRSADGFRLPKEPAKPCILIGPGTGIAPFRSFWQQRLYDLEKKGIK-- 1003
CarpiNOS   NTIKEGDMVPFFAHSSDGFHLPSDPSAPCILVGVGSGIAPFRSFWQQQFHDMKKTGLK-- 1003
BoseNOS    SQLKTGDPVPCFIRGAPSFRLPPDPYVPCILVGPGTGIAPFRGFWQERLHDIESKGLQ-- 1040
PigeNOS    GQLKPGDPVPCFIRAAPSFRLPPDPSLPCILVGPGTGIAPFRGFWQERLHDIESKGLQ-- 1040
DogeNOS    SQLKAGDPVPCFIRGAPSFRLPPDPSLPCILVGPGTGIAPFRGFWQGRLHDIYSKGLQ-- 1040
HeNOS      SQLKPGDPVPCFIRGAPSFRLPPDPSLPCILVGPGTGIAPFRGFWQERLHDIESKGLQ-- 1038
GpigeNOS   SQLKTGDQVPCFIRGAPSFRLPPDPSLPCILVGPGTGIAPFRGFWQERLHDIESKGLQ-- 1041
mouseeNOS  SQLKAGDPVPCFIRGAPSFRLPPDPNLPCILVGPGTGIAPFRGFWQDRLHDIEIKGLQ-- 1037
mousenNOS  NRIQADDVVPCFVRGAPSFHLPRNPQVPCILVGPGTGIAPFRSFWQQRQFDIQHKGMN-- 1273
RatnNOS    NRIQADDVVPCFVRGAPSFHLPRNPQVPCILVGPGTGIAPFRSFWQQRQFDIQHKGMN-- 1273
HnNOS      NRIQADELVPCFVRGAPSFHLPRNPQVPCILVGPGTGIAPFRSFWQQRQFDIQHKGMN-- 1278
RabbitnNOS NRIPADEVVPCFVRGAPSFRLPRNPQVPCILVGPGTAFAPFRSFWQQRQFDIQHKGMS-- 1279
SANOS      ------------------------------------------------------------
```

```
Drosophila ------------------------------------------------------------
mosquito   ------------------------------------------------------------
Rhop       ------------------------------------------------------------
hornworm   ------------------------------------------------------------
Lyms       NTMLSCETTIPSCENSMPSCENTMPSCENTMPSCENTIPSCENTIPSCENTMPSCENTIP 1001
HiNOS      ------------------------------------------------------------
RatINOS    ------------------------------------------------------------
mouseiNOS  ------------------------------------------------------------
GpigiNOS   ------------------------------------------------------------
DogiNOS    ------------------------------------------------------------
ChickeniNOS------------------------------------------------------------
CarpiNOS   ------------------------------------------------------------
BoseNOS    ------------------------------------------------------------
PigeNOS    ------------------------------------------------------------
DogeNOS    ------------------------------------------------------------
HeNOS      ------------------------------------------------------------
GpigeNOS   ------------------------------------------------------------
mouseeNOS  ------------------------------------------------------------
mousenNOS  ------------------------------------------------------------
RatnNOS    ------------------------------------------------------------
HnNOS      ------------------------------------------------------------
RabbitnNOS ------------------------------------------------------------
SANOS      ------------------------------------------------------------

Drosophila ------------------KLPKMWLFFGCRNRD-VDLYAEEKAELQKDQILDRVFLALS 1244
mosquito   ------------------KIPKVWLFFGCRTKN-VDLYRDEKEEMVQHGVLDRVFLALS 1141
Rhop       ------------------KFGKMSLFFGCRLRN-LDLYQEEKESMLKEGILSKVFLALS 1066
hornworm   ------------------KAGHMWLFFGCRHSG-MDLYKDEKEAAVNEGVLTKTRLALS 1096
Lyms       SWERTMQPCQIILPSQTKKHFGEMVLYTGCRTAK-HMIYAAELEEMKRLGVLSNYHVALS 1060
HiNOS      --------------------GGRMTLVFGCRHPEEDHLYQEEMQEMVRKGVLFQVHTGYS 1043
RatINOS    --------------------GGRMTLVFGCRHPEEDHLYQEEMQEMVRKGVLFQVHTGYS 1043
mouseiNOS  --------------------GGRMSLVFGCRHPEEDHLYQEEMQEMVRKRVLFQVHTGYS 1040
GpigiNOS   --------------------GGRMTLLFGCRHPEEDHIYKEEMQEMVQKGVLHEVHTAYS 1045
DogiNOS    --------------------GSRMTLVFGCRRPDEDHLYREEMLEMAQSGVLHEVHTAYS 1043
ChickeniNOS--------------------GGDMILLFGCRHPDMDHIYKEEVEEMKRKGVLKEVFTAYS 1043
CarpiNOS   --------------------GNPVTLVFGCRGSDTDHLYKEETLDMRDNGTLSSITTAYS 1043
BoseNOS    --------------------PAPMTLVFGCRCSQLDHLYRDEVQDAQERGVFGRVLTAFS 1080
PigeNOS    --------------------PAPMTLVFGCRCSQLDHLYRDEVQDAQQRGVFGRVLTAFS 1080
DogeNOS    --------------------PAPMTLVFGCRCSQLDHLYRDEVQDAQQRGVFGRVLTAFS 1080
HeNOS      --------------------PTPMTLVFGCRCSQLDHLYRDEVQNAQQRGVFGRVLTAFS 1078
GpigeNOS   --------------------PAPMTLVFGCRCSQLDHLYRDEVQDAQQRGVFGRVLTAFS 1081
mouseeNOS  --------------------PAPMTLVFGCRCSQLDHLYRDEVLDAQQRGVFGQVLTAFS 1077
mousenNOS  --------------------PCPMVLVFGCRQSKIDHIYREETLQAKNKGVFRELYTAYS 1313
RatnNOS    --------------------PCPMVLVFGCRQSKIDHIYREETLQAKNKGVFRELYTAYS 1313
HnNOS      --------------------PCPMVLVFGCRQSKIDHIYREETLQAKNKGVFRELYTAYS 1318
RabbitnNOS --------------------PCPMVLVFGCRQSKIDHIYREEALQAKNKGVFRELYTAYS 1319
SANOS      ------------------------------------------------------------
```

```
Drosophila REQAIPKTYVQDLIEQE-FDSLYQLIVQERGHIYVCGDVTMAEHVYQTIRKCIAGKEQKS 1303
mosquito   REENIPKTYVQDLALKE-AESISELIMQEKGHIYVCGDVTMAEHVYQTLRKILATREKRT 1200
Rhop       REPSIPKTYVQDLLRVE-CKSVYIQIVQEGGHFYVCGDCTMAEHVFRTLRQIIQDQGNMT 1125
hornworm   REKGIEKKHVQALLEEE-GAEVTRMLLDEEGHFYVCGDCKMAEEVQQKLKFIIKKHAKMT 1155
Lyms       REAALPKMYVQDIIIKN-AAAVYEIVMKKGGHFYVSGDVSMAHDVTRALELVLCQQGGR- 1118
HiNOS      RLPGKPKVYVQDILQKELADEVFSVLHGEQGHIYVCGDVRMARDVATTLKKLVAAKLNLS 1103
RatINOS    RLPGKPKVYVQDILQKELADEVFSVLHGEQGHLYVCGDVRMARDVATTLKKLVAAKLNLS 1103
mouseiNOS  RLPGKPKVYVQDILQKQLANEVLSVLHGEQGHLYICGDVRMARDVATTLKKLVATKLNLS 1100
GpigiNOS   RLPGKPKAYVQDILRQQLAREVLRVLHEEPGHLYVCGNVLMAQDVACTLKQLLAAKLNLN 1105
DogiNOS    RLPGQPKVYVQDILRQQLASQVLRMLHEEQGHLYVCGDVRMARDVAHTLKHLVAAKLSLS 1103
ChickeniNOSRQPGQPKVYVQDILQNELETKVCNILHKEEGHLYVCGDVRMARDVAQTLKRMLVKKLNHT 1103
CarpiNOS   RQTGQPKVYVQDILREQLSDKVFEVLHHNPGHLYVCGGMNMANDVAATIKEILVSRLGIT 1103
BoseNOS    REPDSPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATSVLQTVQRILATEGDME 1140
PigeNOS    REPDSPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATSVLQTVQRILATEGNME 1140
DogeNOS    REPDSPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATSVLQTVQRILATEGDME 1140
HeNOS      REPDNPKTYVQDILRTELAAEVHRVLCLERGHMFVCGDVTMATNVLQTVQRILATEGDME 1138
GpigeNOS   REPNSPKTYVQDILKTELAAEVHRVLCLERGHMFVCGDVTMATNVLQTVQRILASEGDME 1141
mouseeNOS  RDPGSPKTYVQDLLRTELAAEVHRVLCLEQGHMFVCGDVTMATSVLQTVQRILATEGGME 1137
mousenNOS  REPDRPKKYVQDVLQEQLAESVYRALKEQGGHIYVCGDVTMAADVLKAIQRIMTQQGKLS 1373
RatnNOS    REPDRPKKYVQDVLQEQLAESVYRALKEQGGHIYVCGDVTMAADVLKAIQRIMTQQGKLS 1373
HnNOS      REPDKPKKYVQDILQEQLAESVYRALKEQGGHIYVCGDVTMAADVLKAIQRIMTQQGKLS 1378
RabbitnNOS REPDKPKKYVQDILQEQLAEQVYRALKEQGGHIYVCGDVTMAADVLKAVQRIMAQQGKLS 1379
SANOS      ------------------------------------------------------------
```

```
Drosophila EAEVETFLLTLRDESRYHEDIFGITLRTAEIHTKSRATAR-IRMASQP------------ 1350
mosquito   ETEMEKYMLTLRDENRYHEDIFGITLRTAEIHNKSRATAR-IRMASQP------------ 1247
Rhop       DHQVDNFMLAMRDENRYHEDIFGITLRTAEVHNRSRESAR-IRMASQSQP---------- 1174
hornworm   EQEVEEFIFSLMDENRYHEDIFGITLRTAEVHSASREFAKRTRQESLQSQA--------- 1206
Lyms       --EASQQVMSLRDENLFHEDIFGSFVRKAGGQRSEDE---------------------- 1153
HiNOS      EEQVEDYFFQLKSQKRYHEDIFGAVFSYGVKKGNALEEPKGTRL---------------- 1147
RatINOS    EEQVEDYFFQLKSQKRYHEDIFGAVFSYGVKKGNALEEPKGTRL---------------- 1147
mouseiNOS  EEQVEDYFFQLKSQKRYHEDIFGAVFSYGAKKGSALEEPKATRL---------------- 1144
GpigiNOS   EEQVEDYFFQLKSQKRYHEDIFGAVFPHGVKKDRAERPPGDDKL---------------- 1149
DogiNOS    EEQVEDYFFQLKSQKRYHEDIFGAVFPYEVKKDGAAKQPSDPRVPAAHGRS--------- 1154
ChickeniNOSEQQAEEYFFQLKSQKRYHEDIFGAVFPHEVKRI-------------------------- 1136
CarpiNOS   LAQAEEYLSRLKNEKRYHEDIFGS----------------------------------- 1127
BoseNOS    LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERHLRGAVPWAFDPPGP 1200
PigeNOS    LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERHLRGAVPWTFDPPGP 1200
DogeNOS    LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERHLRGAVPWALDPPGP 1200
HeNOS      LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERQLRGAVPWAFDPPGS 1198
GpigeNOS   LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERHLRGAVPWAFDLPGP 1201
mouseeNOS  LDEAGDVIGVLRDQQRYHEDIFGLTLRTQEVTSRIRTQSFSLQERQLRGAVPWSFDPPGP 1197
mousenNOS  EEDAGVFISRLRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DTDEVFSS--- 1429
RatnNOS    EEDAGVFISRLRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DADEVFSS--- 1429
HnNOS      AEDAGVFISRMRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DTDEVFSS--- 1434
RabbitnNOS AEDAGVFISRLRDDNRYHEDIFGVTLRTYEVTNRLRSESIAFIEESKK-DTDEVFSS--- 1435
SANOS      ------------------------------------------------------------
```

```
Drosophila   -----
mosquito     -----
Rhop         -----
hornworm     -----
Lyms         -----
HiNOS        -----
RatINOS      -----
mouseiNOS    -----
GpigiNOS     -----
DogiNOS      -----
ChickeniNOS  -----
CarpiNOS     -----
BoseNOS      DTPGP 1205
PigeNOS      DTPGP 1205
DogeNOS      DTVGP 1205
HeNOS        DTNSP 1203
GpigeNOS     DTSSP 1206
mouseeNOS    EIPGS 1202
mousenNOS    -----
RatnNOS      -----
HnNOS        -----
RabbitnNOS   -----
SANOS        -----
```

Drosophila: drosophila NOS
Mosquito: mosquito NOS
Rhop: Rhodnius prolixus
Hornworm: taboco worm NOS
Lyms: pond snail NOS
HiNOS: human inducible NOS
Rat INOS: rat inducible NOS
MouseeNOS: mouse endothelial NOS
GpigiNOS: guinea pig inducible NOS
DogiNOS: dog inducible NOS
ChickeniNOS: chicken inducible NOS
CarpiNOS: carp fish iNOS
BoseeNOS: bovine endothelial NOS
PigeNOS: pig endothelial NOS
DogeNOS: dog endothelial NOS
HeNOS: human endothelial NOS
GpigeNOS: guinea pig endothelial NOS
MouseeNOS: moue endothelial NOS
MousenNOS: mouse neuronal NOS
RatnNOS: rat neuronal NOS
HnNOS: human neuronal NOS
RabbitnNOS: rabbit neuronal NOS
SANOS: *S.aureus* NOS

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.

Appendix 8 Primers used in *yflM* expression vectors construction

1. ***yflM* primers for cloning to pET11a** :

   5' primer 5' TATGGATCCGTGAAAGACCGTCTCGCG 3'

   3' primer 5' GCGCTCGAGTTAGAACTCTTCCTCATAA GGCTTATCTTG 3'

2. ***yflM* primers for cloning to pET15b and pET21b**:

   5' primer 5' GGGAATTCCATATGAAAGACCGTCTCGCG 3'

   3' mrimer 5'GCGGGATCCTTAGAACTCTTCCTCATAAG GCTTATCTTG 3'

3. ***yflM* primers for cloning to pTrc99a**:

   5' primer 5' TATGGATCCGTGAAAGACCGTCTCGCG 3'

   3' primer 5'CGGAAGCTTTTAATGGTGATGGTGATG ATGCTCATAAGGCTTATCTTGATAAAA ATAGTTCGG 3'

4. ***yflM* primers for cloning to pProEXHT**:

   5' primer 5' GGGAATTCCATATGAAAGACCGTCTCGCG 3'

   3' primer 5' CGGAAGCTTTTAATGGTGATGGTGATGAT GCTCATAAGGCTTATCTTGATAAAAATAG TTCGG 3'

5. ***yflM* primers for cloning to pCWori**:

   5' primer 5' CGCATATGGGAGGACACCACCACCATCA CCACGTGAAAGACCGTCTCGCGGACATT AAAGTGAA 3'

   3' primer 5'GCGCTCGAGTTAGAACTCTTCCTCATAAG GCTTATCTTG3'

6. ***yflM* primers for cloning to pGEX 4T-2 and pCWGST**:

   5' primer 5' ATATCTCGAGTGAAAGACCGTCTCGCG G 3'

   3' primer 5' CGCCTCGAGTTACTCATAAGGCTTATCTTG 3'

Appendix 9 Alignment of SANOS and bovine eNOS.

```
SANOS   ------------------------------------------------------------
bovine  MGNLKSVGQEPGPPCGLGLGLGLGLCGKQGPASPAPEPSRAPAPATPHAPDHSPAPNSPT 60

SANOS   ------------------------------------------------------------
bovine  LTRPPEGPKFPRVKNWELGSITYDTLCAQSQQDGPCTPRRCLGSLVLPRKLQTRPSPGPP 120

SANOS   ---MLFKEAQAFIENMYKECH-YETQIINKRLHDIELEIKETGTYTHTEEELIYGAKMAW 56
bovine  PAEQLLSQARDFINQYYSSIKRSGSQAHEERLQEVEAEVASTGTIHLRESELVFGAKQAW 180
            *:.:*: **:: *.. :   :*  ::**:::* *: .***    *.**::*** **

SANOS   RNSNRCIGRLFWDSLNVIDARDVTDEASFLSSITYHITQATNEGKLKPYITIY---APKD 113
bovine  RNAPRCVGRIQWGKLQVFDARDCSSAQEMFTYICNHIKYATNRGNLRSAITVFPQRAPGR 240
        **: **:**: *..*:*:**** :.  .::: *  **. ***.*:*:. **::   **

SANOS   G-PKIFNNQLIRYAGYDN-----CGDPAEKEVTRLANHLGWKGKGTNFDVLPLIYQLPNE 167
bovine  GDFRIWNSQLVRYAGYRQQDGSVRGDPANVEITELCIQHGWTPGNGRFDVLPLLLQAPDE 300
        *  :*:*.**:***** :      ****: *:*.*. : **.  . .******: * *:*

SANOS   SVKFYEYPTSLIKEVPIEHNHYPRLRKLNLKWYAVPIISNMDLKIGGIVYPTAPFNGWYM 227
bovine  APELFVLPPELVLEVPLGAPHTGVVRGPGLRWYALPAVSNMLLEIGGLEFSAAPFSGWYM 360
        : :::  *..*: ***:   *   :*  .*:***:* :*** *:***: :.:***.****

SANOS   VTEIGVRNFIDDYRYNLLEKVADAFEFDTLKNNSFNKDRALVELNYAVYHSFKKEGVSIV 287
bovine  STEIGTRNLCDPHRYNILEDVAVCMDLDTRTTSSLWKDKAAVEINLAVLHSFQLAKVTIV 420
         ****.**: * :***:**.** .:::** ...*: **:* **:* ** ***:   *:**

SANOS   DHLTAAKQFELFERNEAQQGRQVTGKWSWLAPPLSPTLTSNYHHGYDNTVKDPNFFYK-- 345
bovine  DHHAATVSFMKHLDNEQKARGGCPADWAWIVPPIYGSLPPVFHQEMVNYILSPAFRYQPD 480
        ** :*: .*  .  ** :     ...*:*:.**:  :*.. :*:   * : .* * *:

SANOS   ------------------KKESNANQCPFHH----------------------------- 358
bovine  PWKGSATKGAGITRKKTFKEVANAVKISASLMGTLMAKRVKATILYASETGRAQSYAQQL 540
                       *: :** : .
```

***: designates identical residues;**

: : designates highly conserved residues;

. : designates conserved residues.