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Defenses against Oxidative Stress in *Neisseria gonorrhoeae*: a System Tailored for a Challenging Environment

Kate L. Seib,[1] Hsing-Ju Wu,[2] Stephen P. Kidd,[1] Michael A. Apicella,[3] Michael P. Jennings,[1] and Alastair G. McEwan[1]*

The School of Molecular and Microbial Sciences & Centre for Metals in Biology, The University of Queensland, Brisbane 4072, Australia[1]; Core Facilities for Proteomics Research, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan[2]; and Department of Microbiology and Immunology, University of Iowa, Iowa City, Iowa 52242[3]

INTRODUCTION

Neisseria gonorrhoeae (also known as the gonococcus) colonizes primarily the human genitourinary tract, giving rise to the sexually transmitted infection gonorrhea. Disease caused by this organism is a significant health problem despite continual advances in treatment (24, 25, 87, 250). Worldwide there are an estimated 62 million new cases a year, with an average of 22 million cases at any given time (87).

N. gonorrhoeae inhabits mainly mucosal surfaces of the urethra in males and the cervix in females and as a consequence is exposed to a variety of oxidants, which are generated in three main ways: (i) as a by-product of the bacterium's own metabolic processes, (ii) as a key element of the innate immune response, and (iii) as a result of exposure to other factors within the host environment that promote oxidative stress, such as metal ions or commensal organisms that generate oxidants. The most commonly found and discussed oxidants in biological systems are the reactive oxygen species (ROS), which include the superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\cdot}$), and the reactive nitrogen species (RNS), which include nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$). In addition, sulfur- and chlorine-containing compounds are generated by antimicrobial effector cells (103, 125, 227). Oxidative stress causes damage to DNA, proteins, and cell membranes and often results in cell death (reviewed in reference 125).

Mechanisms for coping with oxidative stress are crucial for the survival of all organisms, particularly obligate human pathogens, such as *N. gonorrhoeae*, that are routinely exposed to oxidative killing by the host and inhabit an environment of unremitting oxidative stress. Defenses against oxidative stress are increasingly being recognized as playing an important role in virulence (28, 67, 102, 105, 123, 206, 251, 258). A greater understanding of the oxidative stress response of microbial pathogens may aid the future development of treatment and

* Corresponding author. Mailing address: The School of Molecular and Microbial Sciences, The University of Queensland, Brisbane 4072, Australia. Phone: 61 7 3365 4655. Fax: 61 7 3365 4520. E-mail: mcewan@uq.edu.au.

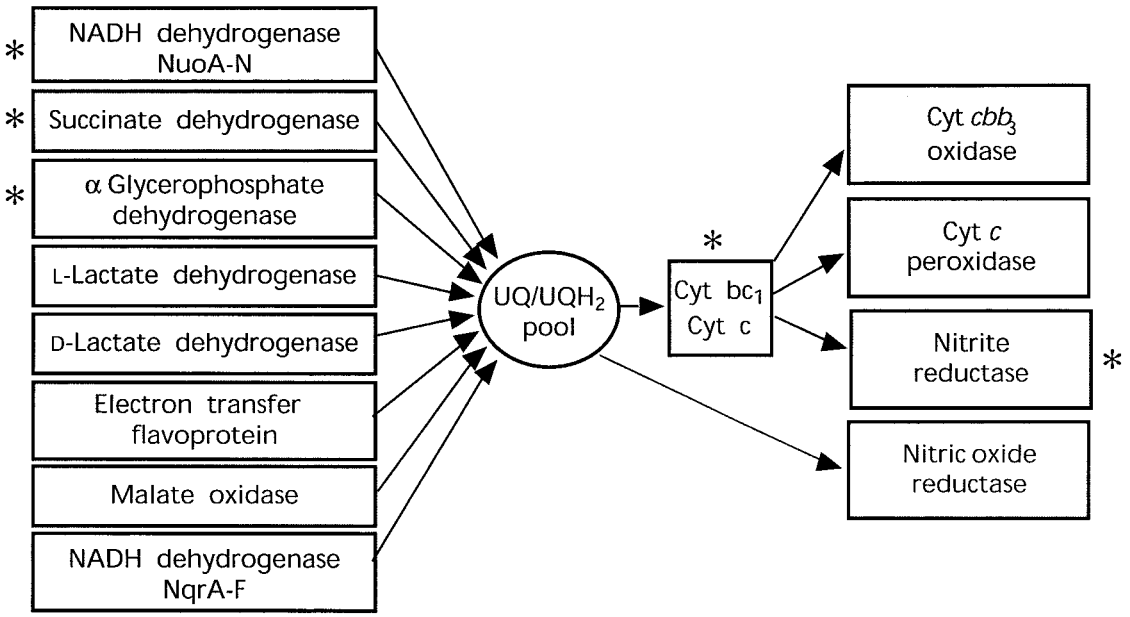

FIG. 1. Electron transport and sources of endogenous ROS and RNS in *N. gonorrhoeae*. Respiratory chains of *N. gonorrhoeae* and *N. meningitidis* are shown, as determined from genomic and biochemical information. Cyt, cytochrome; UQ, ubiquinone. Asterisks indicate potential sites of oxidant generation.

prevention strategies for disease caused by these bacteria. Organisms possess a diverse range of defense mechanisms for sensing, avoiding, and removing oxidants, and *Escherichia coli* is typically used as a model for describing oxidative stress in bacterial systems (reviewed in references 79, 188, 227, and 229). However, the oxidative stress response in *N. gonorrhoeae* differs considerably from the *E. coli* paradigm (235, 236). *N. gonorrhoeae* has evolved to be highly adapted to its environment, and as a consequence it uses a novel combination of regulators and effectors to sense and respond to oxidative stress. An understanding of the mechanism of action of these systems may provide new insights into the biochemistry of oxidative stress defenses that lie outside the *E. coli* paradigm but that may be applicable to many other pathogens.

SOURCES OF OXIDATIVE STRESS

The level and type of oxidative stress that organisms are exposed to depend on the type of bacterium and the environment it inhabits. Oxidants exhibit a broad spectrum of toxic effects in biological systems, with particular species having different reactivities with DNA, proteins, and membranes (125). *N. gonorrhoeae* is exposed to oxidants that are produced both endogenously and exogenously, although some exogenously generated oxidants have the ability to penetrate cells and cause cytoplasmic damage, depending on their membrane permeability (103).

Endogenous Oxidative Stress: the Downside of Aerobic Respiration

High levels of respiration in *N. gonorrhoeae* have long been recognized as a significant potential source of endogenous ROS (9, 133). *N. gonorrhoeae* gives a positive response in the classical oxidase test that is used to distinguish between enteric bacteria and those bacteria that possess a "mitochondrial-type" respiratory chain (27). This means that electron transfer from ubiquinol to oxygen in *N. gonorrhoeae* is catalyzed by a cytochrome bc_1 complex (ubiquinol; ferricytochrome *c* oxidoreductase) and a cytochrome oxidase (ferrocytochrome *c*; oxygen oxidoreductase) (Fig. 1). Unlike almost all other aerobic bacteria, the respiratory chain of *N. gonorrhoeae* has only a single cytochrome oxidase of the cbb_3 type (the CcoNOQP cluster, gene identification no. NG1374 to NG1371; see Table 1, footnote *a*, for the "NG" gene identification website) (176; investigation of available genome sequence [Fig. 1]). The cbb_3-type oxidases are usually characterized by a high oxygen affinity, suggesting that *N. gonorrhoeae* is adapted to microaerophilic growth conditions (186). However, it is well established from research on oxidative stress in mitochondria that the cytochrome oxidase is not a major source of ROS (240).

The major source of endogenous ROS in *E. coli* is a result of auto-oxidation of components of the aerobic respiratory chain (i.e., NADH dehydrogenase [complex I] and succinate dehydrogenase [complex II], as well as fumarate reductase) (124, 167, 168, 178), leading to the generation of 5 μM/s superoxide (124, 126) (intracellular concentration, 10^{-10} M [93]) and 5 to 14 μM/s hydrogen peroxide (208) (intracellular concentration, 10^{-7} to 10^{-6} M [91, 209, 227]). It also seems likely that in *E. coli* the ubiquinol oxidases are also a source of superoxide via reactions involving quinone intermediates. However, since *N. gonorrhoeae* lacks the ubiquinol oxidases found in *E. coli*, eukaryotic mitochondria (99, 149, 164, 241, 259) represent a better comparison for the purpose of identifying sources of oxidative stress arising from quinones. Recent observations have indicated that, in addition to complexes I and II, the cytochrome bc_1 complex (complex III) of the mitochondrial

respiratory chain is a major source of superoxide (99, 149, 164, 241, 259). Since the superoxide is produced as a consequence of the reaction of ubisemiquinol with oxygen at the Q_o site of the enzyme, this means that there is considerable deposition of superoxide into the mitochondrial intermembrane space (104), equivalent to the periplasmic space in a gram-negative bacterium (125). Based on these studies of *E. coli* and eukaryotic mitochondria, NADH dehydrogenase (Nuo), succinate dehydrogenase, and the cytochrome bc_1 complex of the respiratory chain of *N. gonorrhoeae* are likely to be the main sites of endogenous ROS generation (Fig. 1).

Sera from patients with gonorrheal infection recognize both the aerobically induced protein (designated Pox 1) and an anaerobically induced nitrite reductase (AniA), indicating that *N. gonorrhoeae* is capable of both aerobic and anaerobic respiration in vivo (55, 56, 148). *N. gonorrhoeae* uses nitrite (present in cervical fluid at concentrations averaging 28 μM [242]) to support anaerobic growth (55, 143), converting nitrite (NO_2^-) to nitrous oxide (N_2O) in two reactions catalyzed by AniA (153) and nitric oxide reductase (NorB) (6, 120). The nitric oxide produced as a free intermediate during denitrification reactions is a toxic radical species (267). Nitrous oxide has been shown to be the end product of nitrite reduction (153). The genes required for formation of nitrous oxide reductase are present in the gonococcal genome (*nosLYFDZR*, NG1397 to NG1402). However, nonsense mutations in *nosZ*, *nosR*, and *nosD* indicate that an active nitrous oxide reductase is not formed in this bacterium (41).

AniA is a copper-containing nitrite reductase and is the major anaerobically induced outer membrane protein of *N. gonorrhoeae* (26, 55, 115, 165). An *aniA* null mutant is unable to grow anaerobically (119, 165). NorB of *N. gonorrhoeae* is unusual, as nitric oxide reductases are typically two-subunit enzymes encoded by *norBC*. However, as in *Ralstonia eutrophus* (62), no *norC* is seen in pathogenic *Neisseria* species (6, 120). A *norB* mutant cannot grow but can survive anaerobically, suggesting that nitric oxide is not as toxic to *N. gonorrhoeae* as it is to other organisms (120), perhaps as a consequence of redundant systems for defense against RNS (see below).

The toxic nature of nitric oxide requires that the genes involved in its production and removal be tightly regulated and induced only at low oxygen tensions to reduce the coincident generation of nitric oxide and superoxide, which can interact to generate other RNS, such as peroxynitrite (249, 267). In *E. coli*, the transcription factor FNR and the two-component regulatory systems NarQ-NarP and NarX-NarL mediate regulation of nitrate- and nitrite-induced genes (63, 193). Homologues of the *E. coli fnr*, *narQ*, and *narP* genes in *N. gonorrhoeae* have been identified (119, 153). In *N. gonorrhoeae*, AniA synthesis is regulated at the transcriptional level by FNR (114) and is upregulated by NarQ-NarP when nitrite is available (119, 153). NorB is induced by nitrite and nitric oxide but is not regulated by NarP, AniA, or anaerobiosis (120).

Recent in vitro studies, using either nitrite or a nitric oxide donor, have shown that *N. gonorrhoeae* rapidly establishes a nitric oxide steady-state level during anaerobic respiration (41). This organism is able to reduce nitric oxide levels in the surrounding medium from >1 μM (cytotoxic and proinflammatory concentration) to approximately 100 nM (anti-inflammatory concentration) (41).

Interactions of *N. gonorrhoeae* with Host Cells

N. gonorrhoeae infection is usually characterized by a symptomatic localized inflammatory response of the urethra in men (urethritis) (7, 58) and the endocervix in women (endocervicitis) (75). The purulent exudate typical of gonorrhoea contains predominantly activated polymorphonuclear neutrophils (PMNs) (7, 9). The PMN-mediated inflammatory response involves migration of PMNs toward sites of infection, phagocytosis of microorganisms, and elimination of these organisms by oxygen-dependent and oxygen-independent mechanisms (37; reviewed in references 103 and 155). Activation of PMNs results in a rapid increase in oxygen consumption, referred to as the "oxidative burst," which leads to generation of superoxide (16). This species rapidly dismutates to hydrogen peroxide and molecular oxygen (158), and the former is then consumed by myeloperoxidase, generating hypochlorous acid (252). Nitric oxide is also produced by constitutive and inducible nitric oxide synthases of PMNs (44, 146, 160), although the importance of NOS to human PMN killing remains a controversial topic. Various secondary oxidants are generated from these reactive species, including chloramines, hydroxyl radicals, singlet oxygen, and peroxynitrite (44, 103). *N. gonorrhoeae* stimulates the PMN oxidative burst (22, 177, 219, 246). However, interactions between *N. gonorrhoeae* and PMNs have not yet been fully characterized: some studies have reported that *N. gonorrhoeae* is able to survive and replicate within PMNs, while others have reported that *N. gonorrhoeae* is rapidly killed within PMNs (reviewed in references 199, 218, and 219).

In a survival assay using adherent human PMNs, a significant proportion of phagocytosed *N. gonorrhoeae* cells survived PMN killing and replicated over time, in contrast to efficient killing of *E. coli* (219). Investigation of a set of *N. gonorrhoeae* mutant strains, deficient in various oxidative stress defense mechanisms (including superoxide dismutase [SOD] and catalase) and regulatory systems, revealed that none of the oxidative defense enzymes were required on their own for *N. gonorrhoeae* to survive within PMNs in this assay (212). Another study showed that mutant strains lacking the H_2O_2-upregulated genes *recN* (involved in repair of damaged DNA; see below) and NG1686 (a putative zinc metalloprotease) had increased susceptibility to PMN killing relative to the wild type (226). These findings suggest that *N. gonorrhoeae*, like several species of bacteria, may divert the oxidative burst of PMNs (reviewed in reference 3). *Helicobacter pylori* is able to evade PMN killing despite the activation of an oxidative response by targeting of the PMN NADPH oxidase so that it locates to the plasma membrane rather than the phagosomal membrane, thus releasing ROS into the extracellular environment (4). Alternatively, oxygen-independent antimicrobial mechanisms may be of greater significance than oxygen-dependent mechanisms during PMN killing of *N. gonorrhoeae*. In support of the significance of nonoxidative killing, it has been shown that PMNs from people with chronic granulomatous disorder (NADPH oxidase deficient) had phagocytic killing capacities identical to PMNs from healthy donors (198) and that anaerobic PMNs kill as effectively as aerobic PMNs (46). *N. gonor-*

rhoeae cells exposed to serum- or phagocyte-derived lactate have increased metabolism, including increased lactate dehydrogenase activity, and rapidly consume available molecular oxygen, reducing the ability of PMNs to mount an oxidative response (29–31, 82). *N. gonorrhoeae* cells are also susceptible to the oxygen-independent components of PMN granules, including cathepsin G (45, 197, 214–217; reviewed in reference 218).

The presence of a wide range of oxidative stress defenses in *N. gonorrhoeae* may indicate that this organism encounters significant oxidative stress from sources in vivo, in addition to PMNs. The primary sites of gonococcal infection are the ecto- and endocervical epithelia in women (75) and the urethral epithelium in men (7, 58). *N. gonorrhoeae* is able to survive and replicate within epithelial cells at these sites of infection (reviewed in reference 166). Intestinal and airway epithelial cells are able to kill bacteria by oxidative mechanisms (20, 64, 200, 207). There is indirect evidence that cervical epithelial cells may also have an oxidative defense capacity, since it has been observed that several mutant *N. gonorrhoeae* strains that are susceptible to ROS killing in vitro also have decreased survival within primary human cervical epithelial cells. Such mutants include those lacking MntC (a component of the MntABC Mn uptake system) and the oxidative stress response regulator PerR (256), as well as glutathione reductase (Gor) and its regulator OxyR (K. L. Seib, H. J. Wu, Y. N. Srikhanta, J. L. Edwards, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication).

The Host Microenvironment Inhabited by *N. gonorrhoeae*

Several commensal organisms, some of which are a significant source of ROS, coinhabit the genitourinary tract. *Lactobacillus* species, which are part of the normal vaginal flora of most women (77, 113, 163, 195), are believed to control the microflora by production of hydrogen peroxide, bacteriocins, organic acids (e.g., L-lactate) (14, 113), and nitric oxide (173, 257). Hydrogen peroxide-producing *Lactobacillus* species inhibit *N. gonorrhoeae* growth and decrease catalase activity (224, 261). Women with inhibitory strains of lactobacilli are less likely to be infected with *N. gonorrhoeae* (122, 205). Metabolism of L-lactate (produced by lactobacilli) by *N. gonorrhoeae* also greatly enhances oxygen consumption (31), and this in turn may increase levels of endogenous ROS.

DEFENSES AGAINST OXIDATIVE STRESS IN *N. GONORRHOEAE*

Defenses against oxidative stress involve constitutive and tightly regulated adaptive mechanisms to avoid and scavenge oxidants as well as to repair damaged biomolecules. Bacterial responses to oxidative stress are well defined for *E. coli* (reviewed in references 79, 188, 227, and 229). Based on this enteric model system, the generally accepted and simplified paradigm of oxidative stress defenses is that superoxide is removed by SODs (SodA, SodB, SodC), generating hydrogen peroxide in the process, which is removed by catalases (KatE, KatG) and peroxidases (AhpC). However, more than 100 proteins in *E. coli* that are involved in oxidative stress defense have been identified (95, 187). Many of these defenses are controlled by regulators that respond to iron (e.g., Fur), oxygen tension (e.g., FNR and ArcBA), superoxide (e.g., SoxRS), and hydrogen peroxide (e.g., OxyR). The complexity of the regulation of the oxidative stress response in *E. coli* is highlighted by the expression of *sodA*, which is controlled by at least six global regulators (61), and *sodB*, which is controlled by at least three regulators (72). Exposure of *E. coli* to low concentrations of hydrogen peroxide or superoxide induces a protective response that confers resistance to subsequent exposure (79, 127). Similarly, *N. gonorrhoeae* was more tolerant to oxygen when grown previously in hyperbaric pO_2 (9) or when exposed to low sublethal concentrations of ROS (82), indicating the presence of adaptive responses to ROS. The transcriptional response of *N. gonorrhoeae* to hydrogen peroxide has recently been defined; more than 150 genes are differentially regulated after transient exposure to hydrogen peroxide, and 75 of these are upregulated (226).

While *E. coli* serves as a useful model for the oxidative stress response, there are significant differences in the response of *N. gonorrhoeae* to oxidative stress. As a host-adapted pathogen, the oxidative stress response of *N. gonorrhoeae* may represent a more useful model than *E. coli* to help understand the oxidative stress responses of other human pathogens. The current state of knowledge regarding the oxidative stress defenses of *N. gonorrhoeae* is shown in Table 1 and Fig. 2 and is detailed below.

Oxidative Stress Regulons in *N. gonorrhoeae*: OxyR, PerR, and Hydrogen Peroxide

Recent advances in microarray technology and the availability of pan-neisserial genome microarrays have enabled detailed studies of the oxidative stress regulons of *N. gonorrhoeae* to be undertaken. In particular, PerR (256) and OxyR (Seib et al., submitted) regulons have been characterized through the use of *N. gonorrhoeae*/*N. meningitidis* arrays (The Institute for Genomic Research [http://pfgrc.tigr.org/]), and the transcriptome response to hydrogen peroxide (226) has been characterized using pan-neisserial arrays generated by the Neisseria Array Consortium (J. K. Davies and coworkers, personal communication).

Regulation of the peroxide stress response by OxyR is an established feature of gram-negative bacteria such as *E. coli* and *Salmonella enterica* (53, 54). On the other hand, PerR typically regulates peroxide stress responses in gram-positive organisms, including *B. subtilis* (36) and *Staphylococcus aureus* (117). The presence of both PerR and OxyR in a bacterium is rare. Apart from *N. gonorrhoeae*, such a situation has been found only in *Streptomyces coelicolor*, which has OxyR and a hydrogen peroxide-sensitive Fur-like repressor, CatR (100, 101), and *Streptomyces viridosporus*, which contains three peroxide sensor regulatory gene homologues, *ahpA*, *ahpX*, and *oxyR* (194).

Hydrogen peroxide is sensed by OxyR, a member of the LysR family of DNA-binding transcriptional modulators, which is activated by the oxidation of key cysteine residues and formation of a disulfide bond (54, 263). The OxyR regulon of *N. gonorrhoeae* consists of *gor*, *prx*, and *katA* (Table 1 and Fig. 2A; described in detail below). These three genes were differ-

TABLE 1. Defenses and regulators of the oxidative stress response of *N. gonorrhoeae*

Protein name	Function	Gene identification no.[a]	Location(s)[b]	Sensitivity of mutant strain (assay compound[s])[c]	Regulation (details of interest)[d]	Reference(s)
Oxidative stress defenses						
SodB	Superoxide dismutase B	NG0405	C	*x* (PQ, X/XO)	+ (Fur/high Fe, H_2O_2)	9, 226, 236
KatA	Catalase	NG1767	C	*s* (H_2O_2)	+ (OxyR, H_2O_2)	107, 226, 262
MntABC/Mn	ABC-type Mn transporter	NG0168	P, IM	*s* (PQ, H_2O_2)	− (PerR, Mn)	211, 236, 256
Ccp	Cytochrome *c* peroxidase	NG1769	P	*s* (H_2O_2)	+ (FNR/low O_2 + OxyR, H_2O_2)	213, 239[e]
MsrA/B	Methionine sulfoxide reductase	NG2059	C, OM	*s* (H_2O_2, X/XO)	+ (H_2O_2, Ecf σ factor)	221, 226[f]
BfrAB	Bacterioferritin	NG0794, NG0795	C	*s* (H_2O_2, PQ)	NA	49
Sco	Putative thiol:disulfide oxidoreductase/peroxiredoxin	NG1237	ND	*s* (PQ)	NA	211
GSH	Glutathione (GSH synthetase/ glutamate-cysteine ligase)	NG1217, NG0680	C	NA	NA	9
Gor	Glutathione reductase	NG0925	C	*x* (H_2O_2)	+ (OxyR, H_2O_2)	226[e]
Prx	Peroxiredoxin	NG0926	C	*xx* (H_2O_2)	+ (OxyR)	Seib et al., submitted
Laz	Azurin	NG0944	OM	*s* (H_2O_2)	NA	255
PriA	DNA replication restart helicase	NG1437	C	*s* (H_2O_2, CH)	NA	142
RecN	DNA repair	NG0318	C	*s* (H_2O_2)	+ (H_2O_2)	226
NG1686	Putative zinc metalloprotease	NG1686	ND	*s* (H_2O_2, CH)	+ (H_2O_2)	226
NG0554	*N. gonorrhoeae*-specific predicted protein	NG0554	ND	*s* (H_2O_2)	+ (H_2O_2)	226
Oxidative stress regulators						
OxyR	H_2O_2-dependent regulator	NG1813	C	*xx* (X/XO, H_2O_2)	KatA, Prx, Gor	235[e]
PerR	Mn-dependent repressor	NG0542	C	*xx* (H_2O_2)	MntABC, RpmEJ, AdhA	256
Fur	Ferric uptake regulation protein	NG1779	C	NA	Fur, SodB, BfrAB	23, 210
FNR	Fumarate/nitrate reductase regulator	NG1579	C	NA	Ccp	153, 239
NmlR	*Neisseria* merR-like regulator	NG0602	C	*s* (CH, diamide)	AdhC, TrxB, CopA	34

[a] Annotation number from the *N. gonorrhoeae* FA 1090 genome on the Los Alamos National Laboratory website (http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/index.html), from the Gonococcal Genome Sequencing Project of the University of Oklahoma.

[b] C, cytoplasm; P, periplasm; IM, inner membrane; OM, outer membrane; ND, not determined.

[c] Sensitivity of mutant strains to in vitro oxidative stress killing assays relative to wild-type *N. gonorrhoeae*: *x*, same phenotype as the wild type; *s*, sensitive to killing; *xx*, resistant to killing (increased survival relative to the wild type). These assays typically involved exposure of a suspension of 10^4 to 10^6 cells over 1 h to either paraquat (PQ) (10 mM), xanthine (4.3 mM)/xanthine oxidase (300 mU/ml) (X/XO), or hydrogen peroxide (H_2O_2) (10 or 40 mM). Exposure to cumene hydroperoxide (CH) (0 to 1%) and diamide (0 to 50 mM) was performed with liquid cultures over 16 h. For further details, see the references cited in the table. NA, not available. PQ (PQ^{2+}) (1,1′-4,4′-bipyridinium dichloride) is a redox compound that is reduced to the paraquat free radical (PQ^{+}) by low-potential electron donors within the bacterial cell. The paraquat free radical is then oxidized by dioxygen, leading to generation of the superoxide anion ($O_2^{\cdot -}$). This redox cycling also depletes low-potential reducing agents within the cell, such as NADH (106).

[d] Regulation of defenses, either activated/upregulated (+) or repressed (−). Genes of interest, under the control of regulators, are also shown. For details, see the cited references. NA, not available.

[e] Also K. L. Seib, H. J. Wu, Y. N. Srikhanta, J. L. Edwards, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings, submitted for publication.

[f] Also J. K. Davies, personal communication.

entially regulated greater than twofold between the wild type and the *oxyR* mutant strain; *prx* and *gor* were downregulated, and *katA* was upregulated in the *oxyR* mutant strain relative to the wild type. Expression of these genes increases under hydrogen peroxide stress, with OxyR acting as an activator of *gor* and *prx* under high hydrogen peroxide conditions but as a repressor of *katA* under low hydrogen peroxide conditions (Seib et al., submitted). This OxyR regulon is relatively small compared to those of *E. coli* and *S. enterica* serovar Typhimurium, in which OxyR regulates expression of at least nine genes (172, 189, 266) (265). The *E. coli* regulon includes *katG* (hydroperoxidase I), *gorA*, and the peroxiredoxin *ahpCF* (alkylhydroperoxide reductase).

PerR is a member of the Fur (for "ferric iron uptake regulator") family of metalloregulatory proteins (169), and the PerR regulon typically includes antioxidant enzymes such as KatA, AhpCF, and the ferritin-like Dps protein MrgA (110). The PerR regulon of *N. gonorrhoeae* consists of 12 genes that were differentially regulated greater than twofold between the wild type and the *perR* mutant strain (Table 1, Fig. 2A). Eleven genes were upregulated in the *perR* mutant strain relative to the wild type, including the *mntABC* operon, responsible for

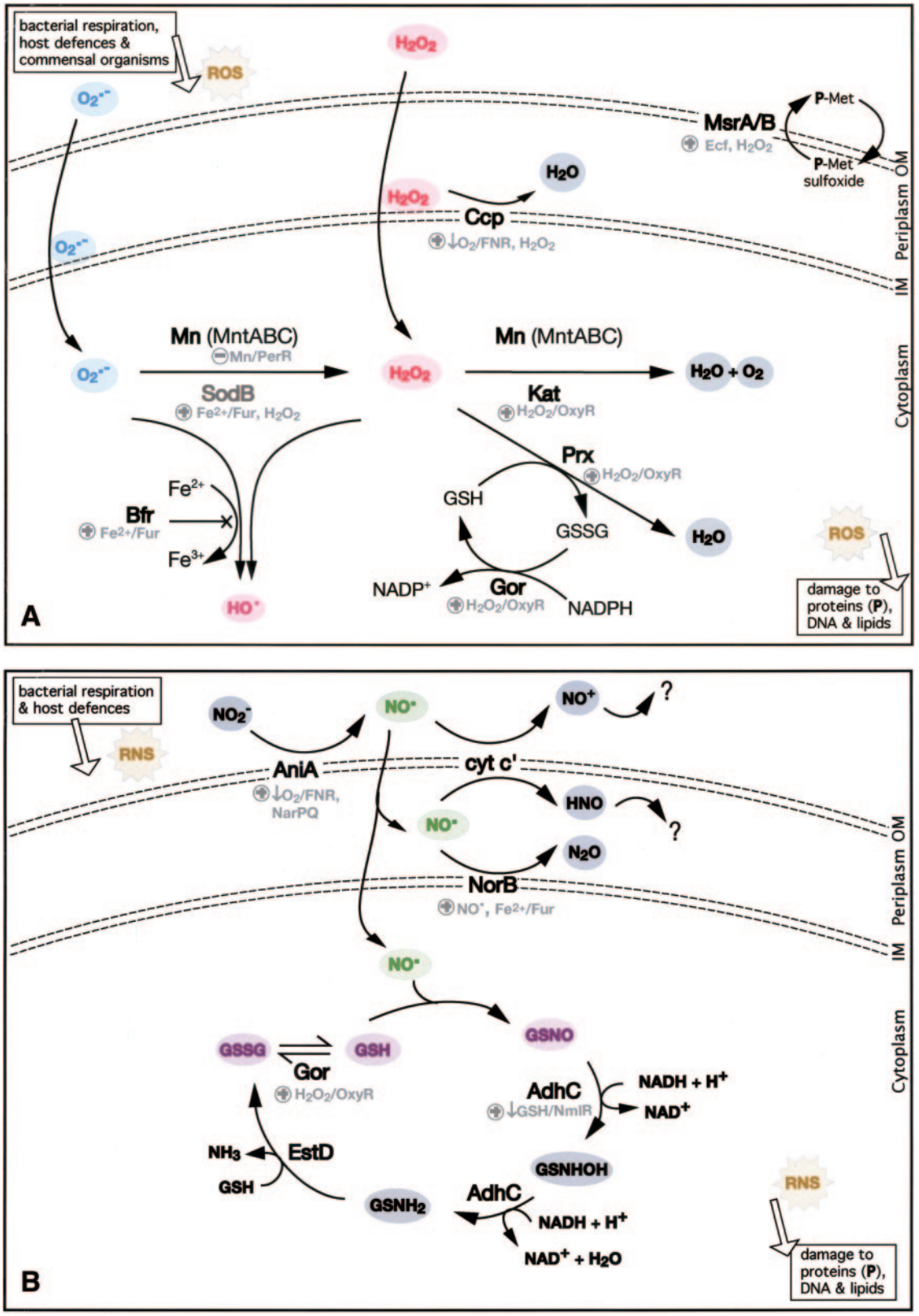

FIG. 2. Oxidative stress responses of *N. gonorrhoeae* to ROS (A) and RNS (B). The following proteins involved in oxidative stress defenses, along with their activities, are shown: MntABC, manganese transporter; Mn, manganese; SodB, superoxide dismutase B; Bfr, bacterioferritin; Kat, catalase; Ccp, cytochrome *c* peroxidase; Prx, peroxiredoxin; Gor, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MsrA/B; methionine sulfoxide reductase; NorB, nitric oxide reductase; cyt c′, cytochrome *c*′; AdhC, glutathione-dependent formaldehyde dehydrogenase; EstD, esterase D. Regulators of these defenses are shown in gray and include FNR, PerR, OxyR, Fur, NmlR, and Ecf. ROS and RNS shown include the following: $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radical; $NO^{\bullet}$, nitric oxide; GSNO, *S*-nitrosoglutathione. Products of the removal of these ROS and RNS include the following: H_2O, water; O_2, oxygen; N_2O, nitrous oxide; NH_3, ammonia. P-Met, protein methionines; +, activation; −, repression; ?, area of uncertainty with respect to certain chemical pathways.

Mn transport (see below); two genes encoding the ribosomal proteins RpmE (L31; NG0930) and RpmJ (L36; NG0931), which have been suggested to have an as-yet-unknown extraribosomal function (157); and two members of the TonB-dependent family (Tdf) of receptors, NG1205 and NG0925 (*tdfH*), which encodes a surface-exposed outer membrane protein with principal homology to heme/hemophore receptors (237). Expression of AdhA (NG1442) is reduced in the *perR* mutant, indicating that PerR acts as an activator of this gene. AdhA has considerable similarity with proteins that belong to the alcohol dehydrogenase subgroup, which contains the NAD(P)- and zinc-dependent alcohol dehydrogenases. Although known regulators of the peroxide defense response are present in *N. gonorrhoeae*, there is no evidence for the presence of SoxR in this bacterium. Thus, the pattern of gene expression in response to superoxide differs considerably from the *E. coli* paradigm.

The transcriptional response to hydrogen peroxide in *N. gonorrhoeae* involves upregulation of 75 genes and downregulation of a further 80 genes (226). Several of the genes that were upregulated encode proteins with a known role in oxidative stress defenses, including catalase, Gor and Prx (OxyR regulon), SodB (Fur regulon; see below), and MsrA/B (sigma factor Ecf regulon; see below). In addition, numerous genes involved in heat shock responses, iron acquisition (including *fur*), and regulation of transcription were shown to be upregulated (226). This study also led to the identification of several new oxidative stress defenses, including RecN (described below), NG1686 (a putative zinc metalloprotease), and NG0554 (an *N. gonorrhoeae*-specific protein of unknown function), all of which are involved in defense against peroxides.

SOD

SOD catalyzes the disproportionation of superoxide to hydrogen peroxide and water (reaction 1) (81, 161):

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (1)$$

Three main classes of SOD exist, SodA (a cytoplasmic Mn-SOD), SodB (a cytoplasmic Fe-SOD), and SodC (a periplasmic Cu/Zn-SOD), all of which are found in *E. coli* (128). *E. coli* mutants lacking SOD have defects in catabolism, biosynthesis, and DNA replication (42, 84, 85).

In early studies it was observed that 80 to 100% of *N. gonorrhoeae* strains had no measurable SOD activity, and the remainder had very low SOD activity (9, 181). This appeared to contradict the accepted view that SOD was essential for all aerobic bacteria. Genes encoding SodA or SodC are not present in *N. gonorrhoeae*, but the gene encoding the complete Fe-dependent SodB (*sodB*) is present in the gonococcal genome (Table 1, Fig. 2A) (236). However, SodB does not appear to be an essential oxidative stress defense of *N. gonorrhoeae* in vitro; a *sodB* mutant showed susceptibility to superoxide killing similar to that of wild-type cells (236). *sodB* expression appears to be increased under iron-replete conditions via positive regulation by Fur (210) (described below), as seen for the similar *sodB* of *N. meningitidis* (97) and *E. coli* (73). Expression of *sodB* is also upregulated after exposure to hydrogen peroxide (226). Thus, the failure to observe superoxide dismutase activity in early investigations may have been due to iron limitation and/or a lack of induction by oxidative stress. The low activity of the Fe-SodB in *N. gonorrhoeae* suggests that the bacterium may be adapted to grow under conditions of iron limitation as a way of minimizing the production of hydroxyl radicals via Fenton chemistry (reaction 2), which would occur if ferrous iron was in contact with hydrogen peroxide:

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\cdot} + OH^- + Fe^{3+} \quad (2)$$

SodB in *E. coli* has a limited role in protection against oxidative stress, which is restricted to the transition from anaerobic to aerobic conditions (135); under aerobic conditions, SodA is of key importance. This type of regulation may be of relevance to *N. gonorrhoeae*, since this bacterium has been proposed to occupy microaerophilic or anaerobic niches (143, 153).

In contrast to *N. gonorrhoeae*, the closely related organism *N. meningitidis* contains active SodB and SodC enzymes (9, 144, 181, 213, 251). Unlike *N. gonorrhoeae*, SodB of *N. meningitidis* has been demonstrated to play a role in protection against oxidative stress (213). The predicted SodB protein sequences of *N. gonorrhoeae* and *N. meningitidis* are 96% identical. The differences seen between *sodB* mutant strains in these two species (213, 236) may be due to distinctive regulatory mechanisms in these organisms. The *sodC* gene of *N. meningitidis* is absent in *N. gonorrhoeae* and appears to have been acquired by *N. meningitidis* via horizontal transfer, most likely from commensal *Haemophilus* species that coinhabit the upper respiratory tract (144, 145). SodC of *N. meningitidis* has been reported to be involved in virulence in a mouse model (251). However, contradictory results have been reported regarding its in vitro role in defense against oxidative stress (213, 251).

Manganese and the MntABC Mn Transporter

N. gonorrhoeae uses the manganous ion (Mn^{2+}) as a chemical quenching agent of ROS in a way similar to the already-established process in *Lactobacillus plantarum*. Reactions 3 and 4 show examples of Mn quenching of ROS (Table 1, Fig. 2A) (11):

$$\begin{gathered} O_2^{\cdot -} + 2H^+ + Mn(II) \rightarrow H_2O_2 + Mn(III) \\ O_2^{\cdot -} + Mn(III) \rightarrow O_2 + Mn(II) \\ \hline 2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2 \end{gathered} \quad (3)$$

$$\begin{gathered} 2H_2O_2 + 2Mn(III) \rightarrow O_2 + 2H^+ + 2Mn(II) \\ 2^{\cdot}OH + 2Mn(II) \rightarrow 2OH^- + 2Mn(III) \\ \hline 2H_2O_2 + 2^{\cdot}OH \rightarrow 2H_2O_2 + O_2 \end{gathered} \quad (4)$$

Lactic acid bacteria lack SOD enzymes but use manganese (Mn) accumulated to millimolar concentrations intracellularly to chemically scavenge superoxide (8, 10–13). In light of the low SOD activity seen in *N. gonorrhoeae* (see above), Mn accumulation is particularly significant. This defense mechanism has also been observed in *Bacillus subtilis* mutants lacking SodA (129). Mn(II) and Mn(III) have been shown to nonenzymatically scavenge superoxide (13) and hydrogen peroxide (13, 223). The rate constant of the interaction of Mn(II) with

the peroxyl radical has been shown to be high enough to provide an antioxidant mechanism (57). In addition, the biologically relevant Mn(II)-pyrophosphate and Mn(II)-polyphosphate complexes can be effective antioxidants by indirectly decreasing or blocking hydroxyl radical production via Fenton, Haber-Weiss, xanthine oxidase-Fe-EDTA, or Fe(III)-H_2O_2-type reactions (51).

Growth of *N. gonorrhoeae* on media supplemented with Mn(II) confers resistance to in vitro oxidative killing by superoxide (236) and hydrogen peroxide (213). This phenomenon is independent of SodB and catalase, respectively, but it is dependent upon an ABC cassette-type Mn uptake system (MntABC) that is involved in this Mn accumulation and protection against ROS (236).

The MntABC Mn transporter of *N. gonorrhoeae* (Table 1, Fig. 2A) belongs to a group of recently characterized ABC permeases that are identified by the nature of the solute that is bound by their extracytoplasmic binding protein component (70). ABC transporters typically consist of three components: a periplasmic (or lipoprotein in the case of gram-positive bacteria) substrate-binding protein (MntC), two integral membrane permeases (MntB), and two peripheral membrane proteins that bind and hydrolyze ATP (MntA) (112). An *mntC* mutant of *N. gonorrhoeae* was shown to have lowered accumulation of Mn and was highly susceptible to superoxide (236)- and hydrogen peroxide (256)-induced oxidative killing compared to the wild type, even in the presence of added Mn. The *mntC* mutant grew at a reduced rate and entered stationary phase later than the wild-type strain, while an *mntAB* mutant strain had a severely defective growth rate, even when grown with Mn-supplemented media (256).

Regulation of *mntABC* expression by Mn and PerR. MntC expression is regulated by PerR (see above) and Mn in *N. gonorrhoeae* (Table 1, Fig. 2A) (256). PerR is a manganese-dependent repressor that regulates the peroxide defense response in gram-positive bacteria such as *Bacillus subtilis* (36, 111) and *Staphylococcus aureus* (117). As mentioned above, although the *perR* gene is largely restricted to gram-positive bacteria, it has been identified in *N. gonorrhoeae* (256) and *Campylobacter jejuni* (243).

An *N. gonorrhoeae perR* mutant strain is more resistant to hydrogen peroxide than is the wild-type strain, and this resistance is enhanced by supplementation of growth media with Mn(II) (256). This is similar to the phenotype of a *B. subtilis perR* mutant strain (36). In the *B. subtilis perR* mutant, catalase, alkyl hydroperoxide reductase, and the DNA binding protein MrgA are all overproduced (50). In *N. gonorrhoeae*, PerR does not appear to have a significant regulatory role in the expression of catalase (256), which is regulated by OxyR in this organism (235) (described below). However, PerR does appear to control Mn accumulation; the *perR* mutant strain has resistance to oxidative killing similar to that of the wild-type strain grown on Mn-supplemented media. Northern and Western analyses indicated that the *N. gonorrhoeae perR* mutant had higher levels of expression of the *mntC* transcript and the MntC protein than did the wild-type strain. A second transcript specific to *mntAB* was also upregulated in the *perR* mutant strain. MntC expression also appears to be repressed by Mn (256).

The regulation of *N. gonorrhoeae* MntABC is distinct from that seen in other bacteria that have been investigated, in which MntABC is regulated by a DtxR-related transcription factor, MntR. In *B. subtilis*, MntR is a Mn-dependent repressor of expression of *mntABC* and other genes involved in Mn uptake (98). MntR is divergently transcribed from *mntABC* loci in *B. subtilis* and a number of other bacteria (36, 118, 192). In *Salmonella enterica* serovar Typhimurium, expression of *sitABC* (an *mntABC* homologue) is repressed by Mn via an MntR homologue but is not responsive to oxidative stress (137). No evidence for the presence of the regulatory gene *mntR* in *N. gonorrhoeae* has been found (256). It has been suggested that the sensitivity of *N. gonorrhoeae* to Mn(II) (236) may be related to the absence of tight control of the MntABC transporter by an MntR homologue (256).

Catalase

Catalases, widespread in aerobic bacteria, are heme-cofactored enzymes that convert hydrogen peroxide to oxygen and water (reaction 5) (162, 208, 209):

$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (5)$$

N. gonorrhoeae possesses very high constitutive levels of catalase (Table 1, Fig. 2A), nearly 100 times higher than *N. meningitidis* (9) and *E. coli* (107). *N. gonorrhoeae* contains a single catalase, encoded by the *katA* gene, that is located primarily in the cytoplasm, with a small concentration potentially located in the cytoplasmic membrane (107, 262). It has been demonstrated that the presence of catalase significantly increases the ability of *N. gonorrhoeae* to resist in vitro killing and DNA damage by exposure to hydrogen peroxide, human PMNs, and *L. acidophilus* (131, 132, 261, 262). A *katA* mutant strain is also significantly more sensitive to hydrogen peroxide and paraquat than is the wild type (213, 222).

Regulation of Catalase: Hydrogen Peroxide and OxyR

The catalase activity of *N. gonorrhoeae* is inducible, increasing two- to threefold when exposed to PMNs or 1 mM hydrogen peroxide (262) and 1.5-fold when exposed to hydrogen peroxide-producing *L. acidophilus* (261). However, this activity is considered weakly inducible (131). Recent analysis of the transcriptome response of *N. gonorrhoeae* to hydrogen peroxide indicated a 51.6-fold increase in levels of *katA* mRNA in cells exposed to 5 mM hydrogen peroxide (226). Expression of catalase is activated by OxyR in *E. coli*, *S. enterica* serovar Typhimurium, and other gram-negative bacteria (53, 172, 189).

A typical OxyR binding site preceding the *katA* gene of *N. gonorrhoeae* has not been found (132, 235). However, it was found that catalase expression is repressed by OxyR (see above) and is induced by hydrogen peroxide via OxyR derepression (Table 1, Fig. 2) (235). An *oxyR* mutant strain has ninefold-higher catalase activity than constitutive levels and fourfold-higher activity than the maximally induced wild-type levels and is significantly more resistant to hydrogen peroxide killing than is the wild type (235). This is distinct from the situation in *E. coli* and *S. enterica* serovar Typhimurium, in which OxyR is a positive regulator of hydrogen peroxide-inducible genes and in which increased sensitivity to hydrogen peroxide is seen in *oxyR* mutants (53, 54). Analysis of

OxyR from *N. gonorrhoeae* indicates that it contains all of the typical features of OxyR proteins. In addition, *N. gonorrhoeae* OxyR can complement an *E. coli oxyR* mutant and behave as an activator (235).

Peroxidase: Cytochrome *c* Peroxidase

Peroxidases, like catalases, are heme-cofactored enzymes responsible for hydrogen peroxide removal (208, 209). Peroxidases oxidize a reductant to convert hydrogen peroxide to water (reaction 6):

$$H_2O_2 + xH_2 \rightarrow 2H_2O + x \quad (6)$$

where x is the reductant.

N. gonorrhoeae has high peroxidase activity, some of which may be associated with the catalase protein (9, 131). However, despite similar catalase concentrations in several *N. gonorrhoeae* strains, different sensitivities to hydrogen peroxide have been seen, indicating additional mechanisms of hydrogen peroxide removal (2).

A periplasmic cytochrome *c* peroxidase (Ccp) in *N. gonorrhoeae* (Table 1, Fig. 2A) (153) that is involved in defense against hydrogen peroxide-induced killing has been reported (213, 239). The *ccp* gene of *N. gonorrhoeae* is located downstream of *katA* (see above), distal to a conserved hypothetical gene (213). An *N. gonorrhoeae ccp* mutant strain showed slight sensitivity to in vitro hydrogen peroxide killing relative to the wild-type strain; however, a *ccp*/*katA* double mutant strain was significantly more sensitive to in vitro hydrogen peroxide killing than was a *katA* mutant strain (213, 239). Ccp belongs to class I of the peroxidase superfamily, along with catalase-peroxidases and ascorbate peroxidases (260). It is a diheme *c*-type cytochrome that catalyzes the reduction of hydrogen peroxide using *c*-type cytochromes of the respiratory chain as the electron donor (reaction 7):

$$H_2O_2 + 2(\text{cytochrome } c_{\text{reduced}}) + 2H^+ \rightarrow 2H_2O + 2(\text{cytochrome } c_{\text{oxidized}}) \quad (7)$$

In *E. coli*, molecular oxygen is sensed by the transcription factor FNR (21). FNR is essential for the gonococcal response to oxygen (153). Ccp of *N. gonorrhoeae* is expressed under conditions of low oxygen tension/anaerobiosis (153) and is dependent on FNR (239). Expression of *ccp* is also upregulated by hydrogen peroxide (226) and PerR (256).

Ccp of *N. gonorrhoeae* is believed to be a lipoprotein, with a signal peptide for cleavage by signal peptidase II, that is anchored to the membrane in the periplasm (239). The lipid modification has been suggested as a way of maintaining the close proximity of Ccp to its *c*-type cytochrome electron donors located in, or associated with, the cytoplasmic membrane, while also maintaining it in a location where its protective mechanism is required (239). However, *N. gonorrhoeae* Ccp may also be involved in energy generation by using hydrogen peroxide as an electron acceptor, as in the yeast *Hansenula polymorpha* (244).

In methylotrophic yeast, Ccp can substitute for catalase. Increased Ccp levels can suppress the phenotype of a catalase-deficient mutant strain (90). In addition, the presence of abundant Ccp activity has been suggested to compensate for the natural absence of catalase in *Fasciola hepatica* and *Schistosoma mansoni*. Partially purified Ccp from these organisms can inhibit damage induced by oxidative stress in vitro (38). Ccp has been identified in several bacteria, including *Pseudomonas aeruginosa* (83), *Pseudomonas stutzeri*, *Paracoccus denitrificans* (92), and *Rhodobacter capsulatus* (121), although functional roles have not yet been determined for Ccp in these organisms. Analysis of the distribution of Ccp in *Neisseria* spp. indicated that it is widespread in *N. gonorrhoeae* and commensal *Neisseria* strains but is absent from all *N. meningitidis* strains (213).

N. gonorrhoeae does not appear to contain the peroxidases present in other bacteria. *E. coli* and *S. enterica* serovar Typhimurium possess an NADPH-dependent alkyl hydroperoxide reductase (AhpC) capable of reducing organic peroxides to alcohols (130, 228). Ahp scavenges the majority of the endogenous hydrogen peroxide in *E. coli* (208). *E. coli* also possesses a thiol peroxidase (47). *N. meningitidis* contains a constitutively expressed cytoplasmic glutathione peroxidase (Gpx) (1, 171) that is involved in oxidative defense (170). An *N. meningitidis gpx* mutant strain is highly sensitive to paraquat and slightly sensitive to hydrogen peroxide (170). However, Gpx is absent from *N. gonorrhoeae* and the commensal *Neisseria* species investigated (171).

Thiol-Based Defenses

GSH and Gor. The low-molecular-weight compound glutathione (γ-L-glutamyl-L-cysteinylglycine) (GSH) is considered one of the first lines of defense against oxidative stress (188). GSH, typically present in cells at millimolar concentrations (5 mM in *E. coli*) (191), is a chemical scavenger of radicals and acts as a hydrogen donor to restore oxidized macromolecules (43). Very high concentrations of GSH (reported to be >15 mM) are present in *N. gonorrhoeae*, which may constitute a powerful antioxidant system (9). The GSH oxidoreductase (Gor) (NG0925), which typically maintains the reduced pool of GSH (43), has recently been identified in *N. gonorrhoeae* as part of the OxyR regulon (Table 1, Fig. 2; described above). As in *E. coli* (266), Gor is upregulated by OxyR under conditions of increased hydrogen peroxide stress (Seib et al., submitted). Gor is annotated in the *N. gonorrhoeae* genome as a dihydrolipoamide dehydrogenase (DldH), due to its similarity to this family of proteins. The *dldH* gene, identified by Stohl et al. (226) to be upregulated by hydrogen peroxide, is the same gene that encodes Gor (Seib et al., submitted).

Thioredoxin and glutaredoxin. The thiol donors thioredoxin (Trx) and glutaredoxin (Grx) are small proteins with conserved cysteine pairs that are oxidized to cystine disulfides upon reduction of cellular proteins. These thiol proteins are members of the cytoplasmic thioredoxin superfamily, the best characterized members of which are thioredoxins 1 and 2 (TrxA and TrxC, respectively) and glutaredoxins 1, 2, and 3 (GrxA, GrxB, and GrxC, respectively) (15). Reduced Trx and Grx are regenerated by an NADPH-dependent thioredoxin reductase and a GSH-dependent Gor, respectively (15). *E. coli* mutants lacking these pathways grow poorly under aerobic conditions (191). Several genes in the *N. gonorrhoeae* genome sequence (156) have been annotated as thioredoxins (NG0652, *trx1*, thioredoxin I; NG1923, *tlpA*, thioredoxin; NG0057, thioredoxin-like protein; NG0331, possible thioredoxin) and glutaredoxins

(NG1381, *grx2*, glutaredoxin 2; NG0114, *grx3*, glutaredoxin 3; NG0351, probable glutaredoxin-related protein). Expression of *trx1*, *grx2*, and *grx3* is upregulated by exposure to hydrogen peroxide (226). A potential thioredoxin reductase (NG0580, *trxB*) has also been identified. Gor of *N. gonorrhoeae* is described above.

Peroxiredoxin. Peroxiredoxins (Prx) are nonheme enzymes that catalyze alkyl hydroperoxide reduction via conserved reactive cysteines, which are typically regenerated by a Trx or Grx thiol reductant (116). The cytoplasmic cofactor NADPH is essential in maintaining the reducing power of the cell for these systems, and enzymes that are involved in maintaining NADPH are upregulated by oxidative stress (187).

A hybrid peroxiredoxin/glutaredoxin (Prx) in *N. meningitidis* that is active in the reduction of various peroxides, including hydrogen peroxide and dehydroascorbate, in the presence of GSH has been isolated (202). The hybrid protein contains a Prx module in the N terminus and a Grx module in the C terminus joined by an eight-amino-acid linker peptide; however, the interaction of these domains remains unclear (202). The Prx protein of *N. gonorrhoeae* has recently been identified as part of the OxyR regulon; its expression is upregulated by hydrogen peroxide-activated OxyR (Table 1, Fig. 2A; see above) (Seib et al., submitted). A *prx* mutant strain has increased levels of catalase and is resistant to hydrogen peroxide killing (Seib et al., submitted).

Sco. A homologue of the yeast Sco (for "synthesis of cytochrome oxidase") protein in *N. gonorrhoeae* was identified and found to be a novel protein involved in oxidative stress defense (Table 1). *N. gonorrhoeae sco* mutant strains were highly sensitive to in vitro paraquat-induced oxidative killing, indicating that Sco is involved in protection against oxidative stress in these bacteria (211).

Members of the SCO1/SenC family (Pfam 02630 [19]) are considered to be involved primarily in biogenesis of the Cu_A center of aa_3-type cytochrome oxidases via an as-yet-undefined role in copper storage or transport (68, 89, 159, 180, 184, 185, 196). However, *N. gonorrhoeae* does not possess an aa_3-type cytochrome oxidase with a Cu_A center (see above), and an *sco* mutant strain is unaffected in cytochrome oxidase activity. It has been proposed that SCO1/SenC proteins have thiol:disulfide oxidoreductase or peroxiredoxin activity and play a catalytic role as an antioxidant protein in the periplasm (52). Primary- and secondary-structure predictions of *N. gonorrhoeae* Sco (211), as well as the recently determined protein structure of the *B. subtilis* Sco protein (17), indicate that these proteins are structurally related to peroxiredoxins and thiol:disulfide oxidoreductases. Thiol:disulfide oxidoreductases and peroxiredoxins are involved in protection against oxidative stress in several bacteria (48, 116, 140).

Methionine sulfoxide reductase. Cysteine and methionine, the two thiol-containing amino acids that occur in proteins, play a role in several antioxidant systems. Methionine residues are highly susceptible to oxidation by superoxide, hydrogen peroxide, and nitric oxide, resulting in formation of methionine sulfoxide residues in proteins (247). Methionine sulfoxide reductase (Msr) is capable of catalyzing the reduction of methionine sulfoxide residues back to methionine (32, 33, 150, 225). *N. gonorrhoeae* produces two forms of Msr, a cytoplasmic form and a form with a signal sequence that is secreted to the outer membrane, which is involved in protection from hydrogen peroxide and superoxide (Table 1, Fig. 2A) (221, 230, 253). Similarly, Msr of *E. coli* is involved in protection from hydrogen peroxide and nitric oxide (174, 225).

MsrA and MsrB, specific for separate methionine sulfoxide epimers, are often separate proteins; however, they are encoded as a fused protein in a single open reading frame in *N. gonorrhoeae*, with an N-subdomain disulfide oxidoreductase, a central MsrA, and a C-subdomain thioredoxin-dependent MsrB (183, 221). MsrA/B was initially designated PilB, a regulator of pilin expression (231), but this has since been disproved (221).

Methionine oxidation has been implicated in conformational changes and inactivation of proteins (247). However, methionine residues are considered endogenous antioxidants in proteins due to the ability to be reduced and regenerated by Msr (150). In *E. coli*, Msr is in turn reduced by the thioredoxin system (225); however, given the location of the gonococcal Msr, the question of how it receives its reducing power is an open one. Msr is also believed to be involved in maintenance of adhesins, which are vital for colonization and virulence, in bacterial pathogens (108, 253).

Expression of *msrA*/*B* is upregulated in response to hydrogen peroxide (226), and the single extracytoplasmic function sigma factor (Ecf) of *N. gonorrhoeae* has also been shown to positively regulate expression of *msrA*/*B* (J. K. Davies, personal communication). The way in which oxidative stress is sensed in this system is not known, but presumably it is controlled by the interaction of an anti-sigma factor and the Ecf protein, as in other systems (reviewed in reference 18).

Azurin

Azurin is a small, blue, copper-containing protein that functions in electron transport during respiration in several different microorganisms (80, 134, 203, 204). Electrons are typically passed through the quinone pool and cytochrome bc_1 and via periplasmic electron shuttles such as *c*-type cytochromes and azurin to nitrite reductase (71, 267). However, azurin of *P. aeruginosa* is not essential for denitrification but is involved in protection from oxidative stress (245). A *P. aeruginosa azu* mutant was proven to be very sensitive to ROS, including hydrogen peroxide and superoxide radicals.

An azurin homologue, *laz* (for "lipid-modified azurin"), has been identified in both *N. gonorrhoeae* (Table 1) (94) and *N. meningitidis* (254). The *P. aeruginosa* azurin is not a lipoprotein (40). Laz is tethered to the outer membrane via palmityl fatty acid (234, 254) and possesses an N-terminal domain consisting of five imperfect repeats of the sequence Ala-Ala-Glu-Ala-Pro (AAEAP), found in the H.8 protein, and a C-terminal domain very similar to that of azurin of other bacteria (94, 136). Laz is not believed to play a direct role in respiration, but may function in an as-yet-unidentified electron transport pathway in pathogenic *Neisseria* species (39). The *N. gonorrhoeae* and *N. meningitidis laz* mutants were more sensitive to hydrogen peroxide, but not superoxide, than was the wild type (255). These mutants also had increased sensitivity to copper toxicity, suggesting that Laz may play an important role in copper sequestration (255).

Iron Sequestration

Iron, although required by aerobic cells, is also problematic due to its ability to catalyze Fenton and Haber-Weiss reactions that generate ROS. Consequently, iron transport is tightly regulated and free iron is maintained at low concentrations within cells (76). In human cells, intracellular iron is sequestered mainly in ferritin while extracellular iron is bound to lactoferrin and transferrin. Bacteria produce scavenging proteins to acquire iron in iron-limited host environments. Intracellular bacterial iron is complexed primarily to ferritin or to the heme-containing bacterioferritin (179). Overloading of bacterial cells with iron leads to accelerated DNA damage (138, 233). Conversely, iron deprivation has been shown to protect *N. gonorrhoeae* from ROS (59).

N. gonorrhoeae is able to use multiple host iron-binding proteins as a direct source of iron, and the organism contains several high-affinity iron storage and transport proteins that maintain low levels of free intracellular iron (reviewed in references 86 and 201). Bacterioferritin (Bfr) of *N. gonorrhoeae*—composed of two subunits, BfrA (ferroxidase center) and BfrB (heme-binding ligand)—is involved in iron storage, growth under iron-limited conditions, and defense against iron-mediated oxidative stress generated by hydrogen peroxide and paraquat (Table 1, Fig. 2A) (49). Most bacteria possess either a ferritin or Bfr homologue (49), although *E. coli* possesses both (5). Investigation of genome databases has not revealed a ferritin homologue in *N. gonorrhoeae* (49).

Regulation of iron uptake by Fur. Microarray analysis has indicated that 203 genes are regulated in response to iron in *N. gonorrhoeae*: 109 genes are upregulated and 94 genes are downregulated by iron deprivation (74). Based on analysis of putative promoter regions, 50 operons were predicted to be directly regulated by Fur (74). The expression of the majority of iron binding proteins is regulated by Fur in *N. gonorrhoeae* (23) in a manner similar to that of several other bacteria (78). The Fur regulon of *N. gonorrhoeae* includes the iron-repressible genes *tbpB*, *lbpB*, *tonB*, and *fbpA*, which are associated with iron sequestration under iron-limiting conditions (74, 86). It has proved more difficult to identify genes under Fur control that are activated in response to high levels of iron. Attempts to generate a Fur null mutant have so far been unsuccessful, which may indicate that Fur plays a critical role in gonococcal survival (232). However, *fur* has since been successfully deleted in *N. meningitidis* (65), and attempts to disrupt Fur in *N. gonorrhoeae* may have failed due to a polar effect on an essential downstream gene. Many iron-responsive genes that have been identified in *N. gonorrhoeae* may not be directly regulated by Fur. However, analysis of *N. meningitidis* has identified at least 15 genes whose levels are increased in the presence of high iron levels and which contain a Fur box in their promoter regions (97). These genes include *sodB*, *recN* (see below), *aniA*, and *norB*. BfrAB (see above) is also potentially regulated by Fur (49).

In *E. coli*, *fur* autoregulates its expression in response to iron levels, but it is also controlled by OxyR and the SoxR/S system and is induced by oxidative stress (264). By lowering iron uptake during oxidative stress, *E. coli* reduces the potential damage that would be caused by Fenton chemistry involving a combination of Fe(II) and hydrogen peroxide. Expression of *fur* does not appear to be regulated by OxyR or PerR in *N. gonorrhoeae*, as indicated by microarray analysis (see below) (256) (Seib et al., submitted). However, *fur* and several of the genes within the Fur regulon are upregulated in response to hydrogen peroxide (226).

The *N. gonorrhoeae* transcriptome response to hydrogen peroxide, described by Stohl and coworkers (226), confirms a link between iron and hydrogen peroxide regulation; however, a more detailed knowledge of individual regulons is required before the pattern of gene expression following the addition of peroxide can be understood fully. This problem is highlighted by the observation that upon the addition of hydrogen peroxide to *N. gonorrhoeae* there was increased expression of genes that are repressed by Fur (iron acquisition genes) and those that are probably activated by Fur (*sodB*, *recN*). Assuming that Fe-Fur is the active form of this transcription factor, it is hard to see how derepression and induction of genes can occur at the same time. It is notable that the collection of data for this experiment involved RNA isolation after a 15-min exposure to hydrogen peroxide (226). As a consequence, there is the possibility that complex changes in gene expression, not to mention changes in mRNA stability, may have been missed in this single-time-point analysis. Of particular relevance here may be changes in the intracellular iron pool in response to oxidative stress. The complex pattern of gene expression in response to hydrogen peroxide highlights the need for investigation of the kinetics of changes in gene expression at a global level and for careful analysis of these regulons.

DNA Repair Mechanisms

It has been suggested that free radicals generated during aerobic respiration are a major source of DNA damage in *N. gonorrhoeae* (142). *N. gonorrhoeae* has a network of DNA repair strategies that includes base excision, nucleotide excision, and mismatch and recombinational repair mechanisms. Comparison of *Neisseria* DNA repair genes with the well-characterized DNA repair mechanisms of *E. coli* has indicated that there are no gross differences in the repair capabilities of these organisms, with the exception of the absence of the SOS response in *Neisseria* (reviewed in reference 141).

PriA, a helicase of *N. gonorrhoeae*, is predicted to deal directly with oxidative stress-induced DNA damage through its role in restarting DNA replication at stalled replication forks (Table 1) (142). A *priA* mutant strain of *N. gonorrhoeae* has decreased DNA repair capability and is sensitive to hydrogen peroxide and cumene hydroperoxide killing relative to the wild-type parental strain (142). RecN, involved in repair of damaged DNA (220), also appears to be directly involved in defense against oxidative stress in *N. gonorrhoeae* (Table 1). A *recN* mutant strain has increased sensitivity to hydrogen peroxide and PMN killing (226). RecN is positively regulated by Fur (described above) (210) and hydrogen peroxide (226).

Defenses against RNS

Bacterial responses to RNS, while not as well characterized as the responses to ROS, are increasingly recognized as being diverse and critical for bacterial survival. In many cases, RNS resistance mechanisms overlap defenses against ROS; for ex-

ample, in *E. coli*, SoxR is activated by both superoxide (96, 152) and nitric oxide (69, 182). The prominent nitric oxide-detoxifying enzymes in *E. coli* appear to be flavohemoglobin (HmpA) and the flavorubredoxin/flavorubredoxin reductase (NorVW) system, regulated by a nitric oxide response regulator, NorR (175). These systems do not seem to exist in *N. gonorrhoeae*. The periplasmic nitrite reductase (NrfA) of *E. coli* has also been shown to reduce nitric oxide (190). This enzyme is a multiheme cytochrome and is distinct from the gonococcal nitrite reductase, AniA (Fig. 2B; see above). Nitric oxide reductase, which catalyzes the reduction of nitric oxide to the less toxic compound nitrous oxide, plays a major role in protecting organisms from nitric oxide (Fig. 2B) (reviewed in reference 267). A knockout mutation of nitric oxide reductase of *P. stutzeri* is lethal, but the effect can be suppressed by a further mutation that inactivates the nitric oxide generator (267). The nitric oxide reductase (NorB) of *N. meningitidis* is able to counteract toxicity due to exogenously added nitric oxide (6). A NorB mutant of *N. gonorrhoeae* is still resistant to nitric oxide (120), indicating that there are additional detoxification pathways present in the bacterium. Cytochrome *c′* (CycP; NG108), an outer membrane lipoprotein that binds nitric oxide in vitro, is believed to protect *N. gonorrhoeae* from endogenously and/or exogenously generated nitrosative stress (Fig. 2B) (238). A *cycP* mutant strain of *N. gonorrhoeae* has an extended lag phase during microaerobic growth in the presence of nitrite. It is suggested that the constitutive synthesis of cytochrome *c′* provides an instant defense by binding nitric oxide until NorB has accumulated to levels sufficient to reduce nitric oxide and by preventing the generation of peroxynitrite from free nitric oxide and superoxide (238). Cytochrome *c′* has also been shown to bind nitric oxide and to act as a buffer against this toxic radical species in *N. meningitidis* (6) and *P. denitrificans* (249). However, cytochrome *c′* may be of limited defense against cytoplasmic nitric oxide.

AhpC is an NADPH-dependent alkyl hydroperoxide reductase, reducing an alkyl (or hydrogen) peroxide to water and alcohol (or water). In both *E. coli* and *S. enterica* serovar Typhimurium, AhpC also acts as a peroxynitrite reductase (35), but no AhpC protein is annotated in the gonococcal genome. However, the bacterioferritin comigratory protein (Bcp; NG0328) of *N. gonorrhoeae* is highly similar in sequence to AhpC of *E. coli* (27% identical and 45% similar over 104 of 167 amino acids) and may be functionally similar. The methionine sulfoxide reductase (MsrA) in the cytoplasm of *E. coli* has also been proposed to be involved in the repair of methionines damaged by peroxynitrite (225), representing an indirect defense against nitric oxide. It is notable that *N. gonorrhoeae* possesses a methionine sulfoxide reductase (MsrA/B; see above) in its outer membrane. Although the gonococcal *msrA/B* mutant was shown to be highly sensitive to oxidative stress, it has not been tested in its sensitivity to RNS (221).

Regulation of RNS defenses. OxyR and SoxRS are the established regulators of the hydrogen peroxide and superoxide responses in *E. coli* (79). Both of these regulators have been shown to also respond to RNS (66, 109). It is worth emphasizing that the mechanisms and roles of these regulators differ among bacterial systems. Indeed, *N. gonorrhoeae* does not appear to possess the SoxRS system, and it is unusual in that its OxyR protein is a repressor of catalase (235) rather than an activator, as in enteric bacteria (54). Although SoxR has been suggested to have a role in nitric oxide sensing, recent microarray experiments with *E. coli* have shown that the response to nitrosative stress is controlled primarily by NorR (the regulator of NorV/W) and Fur (175). *N. gonorrhoeae* lacks the NorR regulator and NorV/W, and this has led to a search for the systems that protect this bacterium against RNS.

Recently, a novel MerR-like transcription factor, NmlR (for "*Neisseria* MerR-like Regulator") (NG0602), which is an activator upon disulfide or redox stress and appears to be a key regulator in the defense against RNS, was identified in *N. gonorrhoeae* (Fig. 2B) (139). NmlR regulates the expression of *adhC* (NG0601), which encodes a zinc-dependent alcohol dehydrogenase (AdhC) that appears to be able to catalyze the reduction of *S*-nitrosoglutathione (GSNO) (Fig. 2A). This enzyme is present in both eukaryotes and *E. coli*, where it has demonstrated activity to remove GSNO (154), although its regulation is different. GSNO is a spontaneously formed adduct between reduced glutathione and nitric oxide; AdhC activity therefore controls the level of *S*-nitrosylated proteins and provides defense against the stresses exhibited by RNS. An *N. gonorrhoeae nmlR* mutant was more susceptible to killing by cumene hydroperoxide and diamide than were wild-type cells and had decreased growth under microaerophilic conditions (139). The *nmlR* mutant is also sensitive to killing by nitric oxide (A. J. Potter, S. P. Kidd, and A. G. McEwan, unpublished observations). The current model for the action of NmlR is that it is a Zn-dependent transcription factor which acts as a repressor of gene expression when it is in the holo form. Upon disulfide stress induced by a variety of electrophiles, such as nitric oxide, aldehydes, or peroxides, it is proposed that NmlR loses Zn and the apo form, which may contain at least one disulfide bridge, acts as an activator of gene expression. This represents a classical MerR-like response mechanism, the switch from a repressor to an activator.

NmlR also regulates a metal ion efflux pump (annotated as CopA; NG0579) and an annotated thioredoxin reductase (TrxB; NG0580) (see above), although the role of these proteins in this novel defense system is not yet defined. Analysis of microbial genomes has defined an NmlR subfamily of the MerR-like regulators, all of which are associated with *adhC*-like genes (139). MerR family proteins do not typically sense redox changes or ROS in the cell, with the exception of SoxR, which contains a superoxide-sensitive iron-sulfur cluster (189).

CONCLUDING REMARKS

Our understanding of the oxidative stress response of *N. gonorrhoeae* has greatly advanced in recent years. This understanding has continuing importance, as gonorrhea remains a serious health risk and has been linked to infertility and increased transmission and susceptibility to human immunodeficiency virus infection (60, 88, 147, 151, 248). Central to this bacterium's ability to persist and cause disease is an ability to overcome the oxidative stress generated in and around the host-pathogen environment.

It has become evident that *N. gonorrhoeae* has evolved complex, and in some cases novel, mechanisms to cope with oxidative and nitrosative stress (Table 1, Fig. 2). The primary defenses used by *N. gonorrhoeae* against ROS include accumu-

lation of manganese by the MntABC transport system (236), unusually high catalase and peroxidase activities (9, 132, 213, 239, 262), and very high concentrations of GSH (9). However, further work is required to better understand the RNS defenses of this organism. Five environmental sensors that are involved in the oxidative stress response have been described to date: the superoxide-dependent OxyR (235), the Mn-dependent PerR (256), the Zn-dependent thiol-based NmlR (139), the iron-dependent Fur (23, 86, 210), and the oxygen-dependent FNR (153, 239). Further characterization of the regulons of these environmental sensors will provide a better understanding of the coordinated response of *N. gonorrhoeae* to oxidative and nitrosative stress.

It is clear that *N. gonorrhoeae* has mechanisms to defend against ROS and/or RNS at the cell surface, in the periplasm, and in the cytoplasm (Fig. 2). *N. gonorrhoeae* has the ability to defend against nitric oxide in these three cellular locations (Fig. 2B). There are no obvious dedicated defense systems against superoxide in the outer membrane, but Sco, which faces the periplasm, may provide some protection against this form of ROS (211). It is interesting that the pK_a for the peroxyl-superoxide interconversion is 4.8; thus, under the acidic conditions of the cervix, the peroxyl radical ($HO_2\cdot$) represents about 50% of the superoxide species outside the cytoplasmic membrane. This neutral form of superoxide is much more permeable across the cytoplasmic membrane, where it forms superoxide by the loss of a proton. Adaptation to a combination of low pH and chronic oxidative stress in the female urogenital tract may be the reason the gonococcus has evolved such an unusual Mn-based defense system against superoxide. It is also interesting that the other superoxide defense system in *N. gonorrhoeae* (SodB) is regulated by iron and does not appear to have a key role in vitro. It is possible that under more iron-replete conditions, SodB would have a key role; it is tempting to speculate that this may be inside a PMN or cervical epithelial cell. Defense against peroxide is also critical, and again it appears that key defenses are associated with the cytoplasm (Fig. 2A). Finally, more work is required to identify the components that provide defense against secondary ROS and RNS products such as peroxynitrite and reactive aldehyde species.

The oxidative stress defenses of *N. gonorrhoeae* have typically been characterized based on the sensitivity of mutant strains to in vitro oxidative killing. However, recent studies using primary human cell lines have revealed interesting findings with respect to the biological significance of several of these oxidative stress responses. A set of mutant strains of *N. gonorrhoeae*, deficient in various oxidative stress defense mechanisms or regulatory systems, was not sensitive to PMN killing in an adherent PMN phagocytosis assay relative to the wild-type strain, despite the stimulation of the oxidative burst by *N. gonorrhoeae* (212). However, examination of the same panel of mutant strains in a primary human cervical epithelial cell line revealed that strains lacking PerR, MntC (256), OxyR, or Gor (Seib et al., submitted) had decreased survival. These findings raise important questions about the major species and sources of oxidative stress that *N. gonorrhoeae* is exposed to during infection. PMN-*N. gonorrhoeae* interactions have a controversial history (reviewed in references 199, 218, and 219), but it has been established that an oxidative burst is stimulated in response to infection by *N. gonorrhoeae* (22, 177, 219, 246). Therefore, it will be interesting to determine how *N. gonorrhoeae* survives PMN oxidative killing, in which several major characterized defenses in vitro do not seem to play a role in vivo. Intestinal and airway epithelial cells are able to kill bacteria by oxidative mechanisms (20, 64, 200, 207), and oxidative stress in urogenital epithelial cells may be a more significant source of oxidative stress than previously recognized. With the lack of relevant animal models, further studies using primary human cell lines may provide answers to some of the questions posed.

ADDENDUM IN PROOF

Recently, we have identified *nmlR* and *adhC* homologues in *Streptococcus pneumoniae* and *Haemophilus influenzae*. An *adhC* mutant of *Streptococcus pneumoniae* and *Haemophilus influenzae* is sensitive to killing by GSNO (S. P. Kidd, M. P. Jennings, and A. G. McEwan, unpublished observations). This contrasts with the situation for *N. gonorrhoeae*, for which an *adhC* mutant shows essentially the same sensitivity to GSNO as wild-type cells. This has led us to conclude either that AdhC is of minor importance in the defense of *N. gonorrhoeae* against NO killing or that there are redundant systems for NO defense in this bacterium. We note that Nikitovic and Holmgren (J. Biol. Chem. 271:19180–19185, 1996) have observed that GSNO can be cleaved by the reduced thioredoxin, generating NO and superoxide. It is interesting that the source of reducing power for thioredoxin, NADPH-dependent thioredoxin reductase, is encoded by a gene (*trxB*) that is also regulated by NmlR. Thus, an alternative route for dealing with GSNO may operate in *N. gonorrhoeae*.

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