New Genes Implicated in the Protection of Anaerobically Grown *Escherichia coli* against Nitric Oxide

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Nitric oxide produced by activated macrophages plays a key role as one of the immune system’s weapons against pathogens. Because the lifetime of nitric oxide is short in aerobic conditions, whereas in anaerobic conditions the cytotoxic effects of nitric oxide are greatly increased as in the infection/inflammation processes, it is important to establish which systems are able to detoxify nitric oxide under anaerobic conditions. In the present work a new set of *Escherichia coli* K-12 genes conferring anaerobic resistance to nitric oxide is presented, namely the gene product of YtfE and a potential transcriptional regulator of the helix-turn-helix LysR-type (YidZ). The crucial role of flavohemoglobin for anaerobic nitric oxide protection is also demonstrated. Furthermore, nitric oxide is shown to cause a significant alteration of the global *E. coli* gene transcription profile that includes the increase of the transcript level of genes encoding for detoxification enzymes, iron-sulfur cluster assembly systems, DNA-repairing enzymes, and stress response regulators.

Macrophages are important weapons of host innate immunity, exhibiting a panoply of concerted strategies for microbial elimination. These strategies include the creation of an environment hostile to bacteria proliferation caused by, among other factors, the production of small diffusible reactive molecules such as nitric oxide (NO). The NO released by eukaryotes is a product of the enzymatic oxidation of L-arginine by NO synthases, and it regulates a plethora of important processes such as signaling, neuronal communication, vasodilatation, smooth muscle relaxation, and inhibition of platelet aggregation. However, these functions are achieved by using low amounts of NO and, once the concentration of NO rises above micromolar levels, the molecule becomes harmful and causes serious deleterious effects, namely tissue inflammation, chronic infection, malignant transformations, and degenerative diseases. Nevertheless, high concentrations of NO are used to fight invading prokaryotic pathogens and parasites. The NO released by macrophages is not the only source of NO that microbes need to deal with, because this compound is also produced abiotically (e.g. by decomposition of nitrite) and biologically by denitrifiers/ammonifiers or other bacteria.

Like all living organisms, bacteria have the ability to respond to aggression by developing a series of not yet fully understood mechanisms that include damage repair, eliciting the SOS system, resistance increase, and use of virulence as a countereffect tactic. Apart from the microbial membrane-bound heme-iron NO reductases, the cytoplasmatic globins, the flavodirion NO reductases, and the multiheme nitrite reductases are also proposed to metabolize NO. *Escherichia coli* contains these three proteins: (i) the flavoredoxin NorV, a flavodirion NO reductase (3), the discovery of which allowed the identification of similar enzymes in a large number of prokaryotic and protozoan genomes (4); (ii) the flavohemogloblin HimPA (5); and (iii) the pentaheme nitrite reductase NrfA (6). Other *E. coli* systems are already known to respond to nitrosative stress, such as the SoxRS regulon. NO binds to the binuclear center of SoxR, forming a dinitrosyl-iron-dithiol active complex that induces soxS. SoxRS mutants were found to increase the sensitivity of *E. coli* to NO produced by macrophages (7). The presence of NO was shown to cause the formation of an iron-nitrosyl species in the *E. coli* Fur (ferric uptake regulator), leading to the inactivation of its repressor activity and thus resulting in a general derepression of the Fur-regulated genes. In a very similar way, nitrosylation of the [4Fe-4S] center of the general oxygen regulator FNR of *E. coli* led to an inactivated form of FNR, which is expected to affect all FNR-regulated genes. To date, inactivation of FNR by NO has only been shown to control *E. coli* himPA regulation (9).

We have first analyzed the variations that occurred in the global gene expression profile of *E. coli* K-12 cells submitted to the deleterious effect of NO under anaerobic conditions, i.e. without the interference of the multiple species resultant from the reaction between oxygen species and NO. NO was found to alter a variety of pathways that altogether result in a change of ~4% of the total transcriptome. Based on these results, a phenotypic analysis of various *E. coli* mutant strains was performed that revealed the existence of new genes involved in NO protection and demonstrated the relevant role of flavohemoglobin for the NO protection of *E. coli* cells grown under anaerobic conditions.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Cell Culture, and Treatment—*E. coli* K-12 (American Type Culture Collection 23716) was used as the wild type strain. Strains LMS2709 (*E. coli* K-12 ΔnorR) and LMS2710 (*E. coli* K-12 ΔnorV) were constructed in collaboration with the group of Prof. J. A. Cole. In these strains, a chloramphenicol resistance cassette replaced a 1.1-kb fragment of norV from 58 bp after the starting codon to 211 bp by guest on July 24, 2018http://www.jbc.org/Downloaded from
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before the end of the gene) and a 1.4-kb fragment of the norR gene (79 bp after the starting codon and 3 bp after the end of the gene). Strain LMS2552 (E. coli K-12 ΔnpaA) was produced by replacing a 1.1-kb fragment of the gene from 44 bp after the starting codon to 14 bp before the end of the gene) with a kanamycin resistance cassette, and strain LMS5262, a double hmpA/norV mutant of the E. coli K-12 strain, was obtained by P1 transduction of strain LMS2552 into strain LMS2710. The strain LMS3711 (E. coli K-12 dydZ) gene was constructed by substituting a 764-bp fragment of the gene (from 99 bp after the starting codon to 220 bp before the end of the gene) with a chaolorphenicol resistance cassette. The LMS4299 strain lacks a 550-bp fragment of the yf/E gene (between 66 bp after the start codon and 150 bp before the stop codon), which was replaced by a chloramphenicol cassette. All of the above-mentioned strains were constructed according to the Wanner and Datsenko method (10). E. coli strains containing single Tn5 insertions in the open reading frames ybdF, ydfU, ydoO, ndrE, ybdD, and yf/E were acquired from the Wisconsin Genome project (www.genome.wisc.edu/functionall/tmmutagenesis.htm).

All cells were grown anaerobically in minimal salts medium, pH 7 (11), at 37 °C and 150 rpm from a 2% inoculum of an overnight aerobic culture in Luria-Bertani medium. Anaerobic conditions were obtained by growing the cultures in rubber seal-capped flasks that, once filled with media and closed, were extensively bubbled with argon. Cultures were grown anaerobically until reaching an A600 of 0.3 at this point, and then unaltered or exposed to NO by means of the injection of an appropriate volume of a 2 mM NO-saturated water solution in order to reach the desired concentration. Preparation of pure NO-saturated stock solutions was performed according to Ref. 12. Prior to saturation with nitric oxide, the solutions (in rubber seal-capped flasks) were depleted from residual oxygen by thorough bubbling with oxygen-free argon, which is preferred to other inert gases because it is heavier than air and will thus delay the diffusion of oxygen into anaerobic vessels. NO was then depleted from contaminants by bubbling through a gas-scrubbing bottle containing 5 mM NaOH; a second scrubbing bottle containing water was used to avoid aerosol contamination. The solutions were saturated with nitric oxide and used immediately.

For the DNA microarray analysis, cells were exposed to 50 μM NO and collected at 4, 8, 16, and 24 h after the addition of NO. NO concentration was determined by using an NO amperometer with a chemically calibrated electrode. For the immunoblotting analysis, cells were exposed to 50 μM NO and collected at 5, 15, 30, 45, or 60 min post NO addition without an extra addition of NO. For the cell growth studies, E. coli wild-type and mutant strains cells were grown until reaching an A600 of 0.3 and submitted to NO exposure. Because it was observed that ~60 min after the bolus of NO, the strains were able to resume normal growth and no effects could be observed, an almost continuous nitrosative stress was achieved by repeating the NO addition five times in periods of 45 min each. The NO concentrations were chosen to cause a visible effect on the growth curve evolution of E. coli K-12 but not enough to resume growth. Furthermore, and despite the fact that physiological NO concentrations remain to be firmly established, the concentrations used are within the range reported by some authors for the in vivo NO concentrations released by macrophages (13–16).

RNA Extraction—Cells were harvested by centrifugation after the addition of a phenol-ethanol mixture that stabilizes bacterial RNA. Total RNA was then extracted from the pellets using the hot acid phenol/chloroform method and treated with DNase I essentially as described at www.microrna.org. Isolated RNA was quantitated on the basis of its absorbance at 260 nm, visualized on an agarose gel to check quality, and stored at −20 °C until further use.

Reverse Transcription-PCR Analysis and Immunoblotting Analysis—The Qiagen® One-Step reverse transcriptase PCR kit was used for the analysis of the selected genes, and the reactions were performed in triplicate using the same RNA samples utilized for the DNA microarray assays. Based on the E. coli K-12 genome sequence, forward and reverse primers were designed for PCR amplification of the following DNA fragments: 110 bp for the gapA gene; 193 bp for the yf/E gene; 362 bp for the narG gene; 497 bp for the hmpA gene; 491 bp for the norW gene; 504 bp for the norR gene; 769 bp for the yf/E gene; and 1082-bp for the ydZ gene. Prior to the reverse transcription PCR experiments, for each pair of primers the optimization of the PCR amplification conditions was performed using an NO amperometer with a chemically calibrated electrode. Sequence analysis of the DNA microarray data was analyzed using the ArrayExplorer software. The intensities were normalized using the LOWESS algorithm for within-gene normalization (19, 18), with the Cy3 channel as the reference and a smooth parameter of 33%. The statistically significant variation of expression in response to the nitrosative stress was obtained by filtering out those genes that had a log(expression ratio) higher or lower than two standard deviations of the average. The microarray data analysis procedures used were fully MIAME compliant.

RESULTS AND DISCUSSION

General Transcription Alterations—RNA extracted from cells of E. coli exposed to NO under anaerobic conditions, a favorable condition for pathogen colonization (23, 24), was used in DNA microarrays to analyze the global gene expression profile alteration caused by nitric oxide. Exponentially and anaerobically grown cells of wild-type E. coli K-12 were left untreated (control) or exposed to 50 μM NO. After 15 min the cultures were harvested by centrifugation, and total RNA was isolated. The amount of NO used was chosen to be sufficient for the induction of genes involved in nitric oxide detoxification without generating growth arrest (see below). Furthermore, working under anaerobic conditions allowed us to obtain a data set free of the effects caused by the combined chemistry of NO and oxygen.

The results obtained from triplicate DNA microarray analyses were normalized and filtered for those genes with statistically significant variations of the transcription levels between control and NO exposed (i.e. log(expression ratio) higher or lower than two standard deviations of the mean value). Of the 4288 genes analyzed, 173 (4% of the genome) passed the filter.
and were further analyzed. These genes were divided into two groups, those that were induced and those that were repressed (90 and 83, respectively; see supplemental tables 1 and 2, available in the on-line version of this article), and divided into functional categories based on TIGR and ECOCYC gene annotations and database searches for identified homologues (Fig. 1). For a subset of genes, reverse transcription-PCR experiments were performed and confirmed the acquired microarray data (Fig. 2).

The affected genes are dispersed through nearly all of the functional categories, the most striking being genes related to virulence functions (7% of the total number of altered genes) (Fig. 1). The general metabolism was clearly affected; energy metabolism was the functional category with the highest number of genes showing significant transcriptional alteration (15%), and genes involved in other metabolisms, such as central intermediary metabolism or cellular processes, had percentages of 9 and 7%, respectively. Another clear effect observed was alteration of the expression of genes involved in transport and binding (13%), which include metal ion transporters and several multidrug or other resistance transporters. A considerable number of genes involved in regulation (7%) were affected, and the same was observed for biosynthesis of cofactors, prosthetic groups, and carriers (6%), in particular those related to iron-sulfur cluster repair machinery and molybdopterin biosynthesis. The largest group of altered genes was, nevertheless, the category of the hypothetical proteins (17%), and from this category were excluded those proteins for which, despite being annotated as hypothetical in the E. coli genome data bank, the BLAST analysis revealed similarity with known proteins; therefore, they were binned in the corresponding category.

Several biosynthetic pathways were affected in response to nitrosative stress exerted onto anaerobically grown E. coli. In particular, NO was found to interfere with the metabolism of pyrimidines, as shown by the decrease in the transcription level of pyrD, pyrI, and pyrB genes that encode enzymes involved in the first steps of the pyrimidine pathway. Also, the nrdHIEF operon involved in de novo biosynthesis of purine and pyrimidine deoxyribonucleosides was induced by NO. Genes related to the maintenance of the cell structure were affected by NO, namely those encoding for the fimbria-like proteins...
ydjO, ydeR, fimG, and ybgQ, which were induced, and flgB, belonging to the flg operon involved in flagellar biosynthesis, which was repressed.

Genes related to the formation of iron-sulfur clusters were induced by NO; these included three genes of the suf operon, namely hscB, encoding the co-chaperone involved in the maturation of iron-sulfur cluster-containing proteins, and iscA and iscR. At the genetic level, the suf and isc operons exhibit important differences, i.e. the suf operon is under the control of both the iron-dependent Fur repressor and the oxidative stress-dependent OxyR activator, whereas the isc operon is auto-regulated (25). NO is a damaging agent of iron-sulfur clusters, and apart from the wide variety of iron-sulfur-containing proteins involved in relevant pathways, the formation of these clusters is essential at least for the transcriptionally active form of regulators such as FNR, IscR, and SoxS (26). Because damage of iron-sulfur centers is a major consequence of nitrosative stress, the induction of isc and suf operons may satisfy the cellular need for iron-sulfur cluster formation.

NO caused inhibition of era transcription, which encodes for the widely conserved GTPase ERA, a multifunctional protein that is involved in cell cycle regulation (by triggering cell division) and protein synthesis (27). NO also induced the transcription of the quorum-sensing regulator sdiA (suppressor of divisio n injection), which controls the expression of virulence factors with a major role in colonization of the enterohemorrhagic E. coli O157:H7 (28). Nitrosative stress caused repression of the fis gene, which encodes for the nucleoid-associated protein Fis, a pleiotropic regulator that is also proposed to regulate virulence genes in bacterial pathogens (29–31).

Resulting from nitrosative stress on the bacterial cells, the SOS response genes (32) such as uscC, cho, and molR-3 were induced, whereas rmuC was repressed. Despite the strong transcription increase of soxS caused by NO, no changes occurred in any of the SoxRS regulon genes, as was also observed for E. coli treated with NO donors under aerobic conditions (33). The existence of inhibitor mechanisms of SoxS by NO cannot be excluded, although it cannot be understood on the basis of the SoxS primary sequence. On the contrary, all of the known Fur-repressed genes were induced, and genes known to be Fur-induced showed a diminished expression. These results are in accordance with the proposed inactivation of the DNA binding capacity of Fur caused by NO (8) and the concomitant abolishment of Fur-dependent regulation. Consistent with the inactivation of FNR by NO (34), it was generally observed that FNR-repressed genes (ndh, hmpA, gpmA, and lpdA) were induced, whereas FNR-activated genes were repressed (narG, pyrD, yhiH, aroP, and rmaC). However, because of overlapping transcriptional regulation some genes failed this trend, as was observed for the FNR-repressed nus operon whose transcription was diminished by NO. Hence, and contrary to SoxRS, Fur and FNR seem to be NO-sensitive regulators resulting in a finely tuned control of their regulons, i.e. they act as multi-responsive regulators.

Although it has been considered that NO needs to be oxidized to react with DNA (35), we observed that upon exposure to NO under anaerobic conditions E. coli activated the regeneration systems, namely by changing the level of DNA repair enzymes. The nrdEF operon codes for the class Ib ribonucleotide reductase, and its expression is tightly co-regulated with genes encoding the accessory proteins NrdH and NrdI (36). Under anaerobic growth conditions, the addition of NO induced transcription of the entire gene cluster nrdHIEF, whereas it was reported that S-nitrated glutathione (GSNO) under aerobic conditions only increased the nrdHI gene expression (33). Interestingly, and in contrast to the other ribonucleotide reductases present in E. coli, the one encoded by nrdHIEF does not respond to inhibitors of DNA replication or DNA-damaging agents that induce the SOS response. To date, it is only known that its expression is triggered by oxidative stress in an OxyR-, SoxRS-, Fis-, RpoS-, cAMP-, or RecA-independent way (36). NO also caused the increase of the transcription of various genes encoding proteins related to multiple resistances, namely ydhK, which encodes a hypothetical protein that contains a fusaric acid resistance conserved region, yafR, far, and ydh, which encode putative bacterial drug-export proteins that mediate resistance to antibiotics, and the operon tehAB, which encodes tellurite resistance proteins (37).

**NO Resistance Behavior of E. coli Mutant Strains**—A set of eight genes that combine a significant NO transcriptional induction with an unknown associated function were analyzed further. To this end, E. coli strains mutated in the target genes were constructed, and the growth behavior of each strain upon the addition of NO was compared with that of the wild type E. coli (Fig. 3). The results showed that the strains mutated on either ydhD, ydhU, ydhO, or nrdE resist NO similarly as does the wild type strain. Furthermore, ydbE and yfjZ exhibited a slightly higher sensitivity, whereas a very strong growth inhibition occurred for strains harboring mutated yidZ and yteE genes. Furthermore, the degree of growth inhibition of the E. coli yteE or yidZ mutated strains is higher than that observed for strains mutated in either flavohemebredoxin or flavohemoglobin (see below). The lack in E. coli of a functional yidZ gene product also resulted in a strain with lower resistance to

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**Fig. 3. Study of the NO resistance of several mutated strains.** E. coli K-12 wild type (wt) and strains mutated in genes ydbE, ydhD, ydhU, ydhO, nrdE, yfjZ, norR, yidZ, and yteE were grown anaerobically in minimal salts medium (filled circles) and submitted to 50 µM NO (open squares) or 150 µM NO (filled triangles) as described under “Experimental Procedures.” The growth curves represent the average of at least three independent measurements.
50 μM NO when compared with the resistance exhibited by the E. coli strain mutated in norR (Fig. 3), the NO-associated transcriptional activator of the norVW genes (38). These results show that yidZ and ytfE play a very important role in bacterial NO protection.

Sequence Analysis of New Proteins Involved into NO Response—The ytfE gene is predicted to encode for a cytoplasmic protein, and a BLAST search using the E. coli K-12 YtfE (Ec_YtfE) sequence retrieved a number of highly similar sequences from enterobacteria, several sequences (mainly from pathogens) annotated as regulators of cell morphology and NO sensing (RCMNS), and also Ralstonia eutropha NorA and Psedomonas stutzeri DnrN, both shown to be affected by NO in terms of their regulation. NorA is inserted in the Nor operon, coding for the respiratory NO reductase from R. eutropha, and the product of this gene does not appear to be essential for denitrification, because a mutant carrying a deletion on it showed no alterations in its phenotype when grown in denitrifying conditions (39). DnrN was also shown to be regulated by NO, but its function is not yet known (40). More recently, the YidE homologue from Salmonella enterica serovar Typhimurium was identified as one of the gene products from this organism to be induced by acidified nitrite, which leads to the production of NO (41). Nevertheless, the role of this protein remains elusive, as a mutant deleted in its gene does not display any growth defects under these conditions (acidified nitrite). The present work shows that in E. coli ytfE is induced by NO, having the highest NO transcriptional level among all genes. Moreover, a severe growth impairment of a YtfE knock-out mutant under nitrosative stress was observed, clearly showing that YtfE is of major importance in the response of E. coli against NO. YtfE also shows 26% identity with Staphylococcus aureus ScdA, a protein proposed to be involved in cell wall physiology (42). However, E. coli cells mutated in ytfE did not present different morphological characteristics when compared with the wild type E. coli K-12 (data not shown).

An amino acid sequence alignment (Fig. 4), performed with YtfE from enterobacteria, several RCMNS proteins, NorA, and DnrN, reveals a series of conserved motifs throughout the whole protein sequence. Sequence identity within the enterobacterial YtfE ranges from 89 to 100%, RCMNS proteins share 48–61% identity with Ec_YtfE, and both NorA and DnrN also have a high degree of identity with Ec_YtfE (48 and 46%, respectively). Among the conserved motifs and residues, a highlight feature is the number of conserved carboxylate (aspartate and glutamate) and histidine residues (see “Results and Discussion”).

Fig. 4. Amino acid sequence analysis of E. coli YtfE (Ec_YtfE). Shown is sequence alignment between Ec_YtfE, other enterobacterial YtfE proteins, proteins annotated as regulators of cell signaling, and NorA and DnrN, produced with Clustal X, version 1.8 (21). Sf_YtfE, Shigella flexneri YtfE (30065498); uEc_YtfE, E. coli O157:H7 YtfE (15804800); St_YtfE, Salmonella typhimurium LT2 YtfE (16767645); Er_YtfE, Erwinia carotovora (50122527); Hp_YtfE, Yersinia pestis RCMNS (45440406); Hi_YtfE, Haemophilus influenzae RCMNS (42290252); Ms_YtfE, Mannheimia haemolysans RCMNS (52307350); R rcYtfE, R. eutropha RCMNS (46131630); Re_NorA, R. eutropha NorA (52551677); Re_DnrN, R. eutropha DnrN (54363021); Sm_NorA, S. marcescens NorA (16767645); Sm_NorB, S. marcescens NorB (16767645); Sm_YidZ, S. marcescens YidZ (16923108). Secondary structure of Ec_YtfE was obtained from the PSIPRED server. Full line, α-helix; block arrows, β-sheets; dashed line, β-strands; wavy line, reverse turn.
the LysR family of regulators, it only shows 20–27% of sequence identity with the remainder YidZ and LysR regulators. The secondary structure of *E. coli* YidZ was predicted using the PSIPRED server, and an attempt was made to model its tertiary structure using as templates the retrieved structures of DntR (Protein Data Bank code 1UTH) and CbnR (Protein Data Bank code 1IXC), two members of the LysR family whose crystallographic structures were solved (45, 47). In the latter procedure, only a small segment of the sequence was modeled; interestingly it was the part corresponding to the helix-turn-helix DNA-binding domain of LysR-type regulators. The C-terminal region, which displays a lower degree of similarity, has its variability correlated with the ability of LysR-type regulators to bind a multitude of different inducers. Overall, the observations regarding the sequence of YidZ place this gene product in the family of LysR-type regulators; all that remains is to clarify the specific cellular function(s) that should be assigned to it. Our results clearly show that its role is related to nitrosative stress response, either through the repair of a cellular lesion or by being involved in the regulation of the detoxifying systems.

**Comparative Analysis of E. coli NO Detoxification Systems**

The microarray data showed that anaerobic nitrosative stress caused a strong induction of the transcriptional levels of *norVW* operon and *hmpA* genes, in accordance with previous observations (11, 34). In addition, the data strongly suggest that flavohemoglobin is able to protect *E. coli* against NO in anaerobic conditions. In fact, we observed that the anaerobic growth of *E. coli*/H9004 hmpA was impaired in the presence of NO (Fig. 6). Furthermore, not only did the *E. coli*/H9004 hmpA and *norV* strains behave similarly, but, more importantly, NO was found to cause a severe growth arrestment of the *E. coli* *norV*/H9004 hmpA
double mutant strain. These results show the prominent role of HmpA in anaerobic NO detoxification and indicate that the protection against NO is achieved by means of both flavorubredoxin and flavohemoglobin. These data are in agreement with previous proposals (48, 49) but contradict the recent proposal of Gardner and Gardner that flavohemoglobin detoxifies NO only under aerobic conditions (50).

The correlation between mRNA level and protein abundance was assessed by immunoblotting analysis. To this purpose, exponentially growing cells of E. coli, raised in anaerobic conditions and submitted to 50 μM NO, were collected at different intervals of time ranging from time 0 (cells not exposed to NO) to 1 h after nitrosative stress induction and analyzed. The results revealed that flavorubredoxin and HmpA have quite different protein profiles (Fig. 7). Flavorubredoxin expression occurs immediately after the addition of NO (5–15 min) and remains approximately constant afterward. In contrast, HmpA expression is maximal much later, at ~45 min. These results suggest that the faster response is achieved by the enzyme that, in vitro, has the higher NO reductase activity, i.e. flavorubredoxin. Furthermore, analysis of the expression of flavorubredoxin in the E. coli ΔhmpA strain and the expression of flavohemoglobin in the E. coli ΔnorV strain showed that the absence of one of the proteins did not imply an increase in the protein amount of the other, i.e. no compensatory effects occur in the mutant strains.

Conclusion—The transcriptome obtained from DNA microarray analysis of anaerobically grown E. coli cells exposed to nitric oxide showed that NO triggers the alteration of the expression of genes dispersed throughout the chromosome that encode for proteins involved in a wide range of cell functions, including yet uncharacterized ones. In addition, the transcriptional profile revealed multiple strategies for the bacterial survival through the induction of several enzymatic systems, including the following: (i) detoxification enzymes; (ii) iron-sulfur cluster assembly systems; (iii) DNA-repairing enzymes; (iv) stress response regulators; and (v) possible metabolic changes to increase cell energy production and inhibit nitrite oxide synthase. In particular, it was observed that the effect of NO on its main target, the iron-sulfur proteins, is counterbalanced by a considerable increase in the transcription of the genes for the assembly machinery (suf and isc). DNA is also a target of NO; in accordance, several enzymes involved in DNA repair and/or de novo biosynthesis of nucleic acids were induced.

NO is known to affect several regulons, namely those that involve iron proteins as transcriptional regulators (Fur, FNR and SoxR). Therefore, a quite complex regulatory network becomes activated upon NO exposure, causing a significant change in the transcription levels of multiple genes. However, many of the genes responsive to these regulators are also regulated by many other proteins, such as the nucleoid proteins (e.g. Fis) (51), adding an extra complexity to the transcriptional behavior. Whereas the effect on Fur and FNR caused transcription alterations in the genes under their control, that of SoxR was unexpected; although a large increase upon the transcription of soxS occurred, a corresponding increase on the SoxRS regulon could not be detected.

The present work revealed two new genes involved in anaerobic NO protection of E. coli, YidE and the potential regulator YidZ. Furthermore, it showed that flavohemoglobin, despite its lower in vitro turnover for NO reduction compared with that of flavorubredoxin (3, 52), confers a similar degree of anaerobic NO protection to E. coli, and the absence of these two genes caused a severe growth impairment as judged by the results observed for the E. coli ΔhmpAΔnorV double mutant. It should be mentioned that establishment of the oxygen-related conditions in which E. coli HmpA is able to work is most important, because genes encoding for flavohemoglobin are present in a wide range of genomes of organisms, including those of several protozoa (2). Furthermore, we observed that only nine up-regulated genes (sufA, hmpA, nrdH, nrdI, norV, norW, ilvC, soxS, and yidE), ~10% of the total number of genes induced by NO under anaerobic conditions, fit the gene profile of E. coli exposed to NO donors in an aerobic environment (33), showing that the conditions chosen to study the effects of NO are of crucial importance. The fact that pathogen colonization occurs in close to anaerobic environments and the fact that many protozoan pathogen systems are analogous to anaerobic organisms point to the relevance of using anaerobic conditions for the study of pathogenic NO resistance mechanisms.

Analysis of the E. coli transcriptome under the studied conditions revealed that the protective NO mechanisms share some degree of similarity with other stress responses, including antibiotic resistance. The induction by NO of genes involved in the assembly of iron-sulfur clusters (iscRSUA and sufAB) was also observed under H2O2 (53). The induction of soxS by NO is common to oxidative stress caused by superoxide (54) or H2O2 (55) and by antibiotic stress (4,5-dihydroxy-2-cyclopenten-1-one) (56). The down-regulation of the pyridine biosynthesis (pyrD repression) and the induction of the nrdHIEF operon, the molybdenopterin biosynthesis genes (mopaB and mocaC), and the tellurite resistance gene (tehAB) were also detected in the gene expression profile of E. coli treated with antibiotics (56).

In summary, NO induces global changes on the metabolism of E. coli that must now be the subject of specific studies and that ultimately may give rise to the development of new microbicidal agents, alternatives to the β-lactam-derived antibiotics, which are coming to a halt.

Acknowledgments—We thank Prof. J. A. Cole for collaboration on the construction of the E. coli norV and norR mutant strains, Prof. R. Poole for providing the rat polyclonal antiseraum against HmpA, and Dr. J. M. Santos (STAB Vida) for technical support.

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New Genes Implicated in the Protection of Anaerobically Grown *Escherichia coli* against Nitric Oxide

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*J. Biol. Chem.* 2005, 280:2636-2643.

doi: 10.1074/jbc.M411070200 originally published online November 16, 2004

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