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Review

A tale of two sites: how inflammation can reshape the microbiomes of the gut and lungs

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RECEIVED MARCH 2, 2016; REVISED MAY 24, 2016; ACCEPTED JUNE 6, 2016. DOI: 10.1189/jlb.3MR0316-106R

ABSTRACT

Inflammation can directly and indirectly modulate the bacterial composition of the microbiome. Although studies of inflammation primarily focus on its function to negatively select against potential pathogens, some bacterial species have the ability to exploit inflammatory byproducts for their benefit. Inflammatory cells release reactive nitrogen species as antimicrobial effectors against infection, but some facultative anaerobes can also utilize the increase in extracellular nitrate in their environment for anaerobic respiration and growth. This phenomenon has been studied in the gastrointestinal tract, where blooms of facultative anaerobic Gammaproteobacteria, primarily *Escherichia coli,* often occur during colonic inflammation. In cystic fibrosis, *Pseudomonas aeruginosa*, another Gammaproteobacteria facultative anaerobe, can reduce nitrogen for anaerobic respiration and it blooms in the airways of the chronically inflamed cystic fibrosis lung. This review focuses on the evidence that inflammation can provide terminal electron acceptors for anaerobic respiration and can support blooms of facultative anaerobes, such as *E. coli* and *P. aeruginosa* in distinct, but similar, environments of the inflamed gastrointestinal and respiratory tracts.
***J. Leukoc. Biol.* 100: 943–950; 2016.**

Introduction

The host immune system is often considered metaphorically to be "at war" with microorganisms, in which the immune system mounts a host defense against an invading army of potential pathogens. In this context, inflammation is thought of as a weapon that produces activated cells and antimicrobial molecules to destroy the invaders. This metaphor would work if the body during health were sterile; however, it is not. In actuality, complex communities of microorganisms live on every surface area of the human body during health and those communities change, but still persist, during inflammatory responses. Even areas of the body once considered sterile, such as the lungs, are now known to harbor low levels of bacteria during health [1, 2].

Certain bacteria, primarily those found in the Proteobacteria phylum, contain the metabolic capacity to utilize inflammatory byproducts for their survival [3]. During chronic inflammation these Proteobacteria "bloom" (*i.e.,* increase in relative abundance) within a microbiome and outcompete bacteria that lack the metabolic capacity to benefit from inflammation. Although this connection between inflammation and Proteobacteria bloom has been recognized in the gastrointestinal microbiota literature, we are only beginning to appreciate that the same mechanisms may apply to the respiratory microbiota and the bloom of *Pseudomonas aeruginosa* during CF and potentially other chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease.

GASTROINTESTINAL INFLAMMATION AND THE OUTGROWTH OF GAMMAPROTEOBACTERIA

Large-scale disturbances of the bacterial community composition in the GI tract can affect the health of the host, a phenomenon known as "dysbiosis" [4–6]. In a state of health, the GI tract contains a large, complex microbiota that is dominated by obligate anaerobes from the phyla Bacteroidetes and Firmicutes, with members of the phyla Proteobacteria and Actinobacteria occurring in lower numbers. Antibiotics, diet and injury/changes in the epithelium can result in dysbiosis. One increasingly recognized factor contributing to dysbiosis in the gut is the onset of inflammation. During GI inflammation, the obligate anaerobes of the Bacteroidetes and Firmicutes phyla decrease in relative abundance, whereas Proteobacteria increase to become the most prominent phyla in the gut, largely attributable to outgrowth of Gammaproteobacteria, which include the Enterobacteriaceae.

Abbreviations: CF = cystic fibrosis, CFTR = cystic fibrosis conductance regulator, GI = gastrointestinal, IBD = inflammatory bowel disease, KEGG = Kyoto Encyclopedia of Genes and Genomes, MAMP = microbe-associated molecular patterns, N_2 = dinitrogen, NO_2^- = nitrite, NO_3^- = nitrate, O_2^- = superoxide, $ONOO^-$ = peroxynitrite, ROS = reactive oxygen species, RNS = reactive nitrogen species

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A bloom of Enterobacteriaceae in response to GI inflammation has been demonstrated in numerous contexts. In both infection-induced and genetic mouse models of IBD, microbiota dysbiosis in response to inflammation is marked by a large relative increase in Enterobacteriaceae [3, 7–18]. This finding has been demonstrated for *Escherichia coli, Salmonella typhimurium*, and *Klebsiella pneumoniae*. Individuals with IBD are known to have an altered gut microbiota marked by an increased relative abundance of Enterobacteriaceae, as well as other members of the Gammaproteobacteria class and Proteobacteria phylum [19–24]. These clinical and experimental observations support the concept that some aspect of Gammaproteobacteria physiology provides them with a growth advantage over other members of the indigenous microbiota on the inflamed GI mucosa.

BACTERIAL ANAEROBIC RESPIRATION IN THE GI MUCOSA

The healthy colon is an anaerobic environment and the energy needs of the bacteria in the GI microbiome are met primarily through fermentation. The availability of specific terminal electron acceptors in the environment is a major factor influencing the composition of the bacterial microbiome. In the absence of inflammation, the GI mucosa lacks sufficient quantities of molecular oxygen or NO_3^- for Enterobacteriaceae to carry out oxidative phosphorylation via the electron transport chain, thereby limiting the growth of these organisms.

In sharp contrast, it has recently been recognized that inflammation changes the metabolic environment of the mucosa by providing the terminal electron acceptors needed for anaerobic respiration by Enterobacteriaceae, thereby allowing them to outgrow (or "bloom") the other resident anaerobes that primarily use fermentation for their energy needs (recall that fermentation generates less ATP than respiration) [3, 25]. One of the central antimicrobial actions of inflammatory host cells is the production of ROS, primarily in the form of O_2^-, and RNS, primarily in the form of NO [26]. O_2^- and NO can react together to produce $ONOO^-$, which decomposes into NO_3^- and (NO_2^-) (for a detailed description of the reaction, please see ref. 27). Extracellular NO_3^- can be used as a terminal electron acceptor to support anaerobic respiration and growth of Gammaproteobacteria in vivo via denitrification [3, 7–13].

RNS produced via iNOS in myeloid cells during inflammatory reactions have long been held to play a major role in the antimicrobial activity of these cells [28–37]. Antimicrobial activity can be mediated by iNOS and RNS directly, as well as induction anti-microbial peptide expression and innate immune system signaling. However, iNOS/RNS can also promote the colonization of inflamed mucosal surfaces by providing a selective advantage for denitrifying facultative anaerobic bacteria, such as *E. coli*, *S. typhimurium*, *K. pneumoniae* and most Gammaproteobacteria, including *Pseudomonas aeruginosa* [3]. NO_3^- reductase activity is critical for the utilization of NO_3^- as a terminal electron acceptor. Winters et al. [3] performed comparative genomics to determine the predicted presence of NO_3^- reductase activity across different bacterial phyla. As shown in **Fig. 1** (reprinted from their study [3]), members of the Gammaproteobacteria had the highest prevalence of NO_3^- reductase activity, with the highest percentage found in the Enterobacteriaceae. In contrast, members of the Bacteroidia and Clostridia classes, which are obligate anaerobes and the major members of the gut microbiome in the absence of inflammation, have low to no NO_3^- reductase activity. This genomic data has been used to support the model that the availability of host-provided NO_3^- promotes the bloom of Gammaproteobacteria observed in inflamed anaerobic mucosal sites [3, 25].

EXTRACELLULAR NO_3^- AND BACTERIAL ANAEROBIC RESPIRATION

E. coli has been shown to utilize NO_3^- provided through host inflammation for growth through nitrogen respiration [13, 38]. *E. coli* is able to reduce NO_3^- to ammonium through a 2-step dissimilatory NO_3^- reduction pathway (KEGG: http://www.genome.jp/kegg-bin/show_pathway?org_name=eco&map-no=00910&mapscale=&show_description=hide). As displayed in **Fig. 2**, NO_3^- is first reduced to NO_2^- by NO_3^- reductase, followed by reduction of NO_2^- to ammonium by NO_2^- reductase. Nonpathogenic *E. coli* strain MG1655 displays increased in vitro growth under low-oxygen conditions if exogenous NO_3^- is provided [38]. In the same study, NO_2^- and ammonium was shown to increase proportionally with NO_3^- supplementation but decreased disproportionally with O_2 concentration. The NO_3^- reductase enzymes all require a molybdenum cofactor in the active site to function, and this has been used to demonstrate the requirement for NO_3^- reductase. An *E. coli* mutant strain that lacked the molybdenum binding site in the NO_3^- reductase enzyme (*moaA*) was outcompeted by the wild-type *E.coli* strain in NO_3^--supplemented cultures [13]. In 2 different mouse models of intestinal inflammation, NO_3^- levels were increased in the cecal mucus layer during inflammation, and wild-type *E. coli* also outcompeted *moaA* mutant strains. Similar results were seen for NO_3^- reductase deletion mutants. Most strikingly, since iNOS is the sole source of NO_3^- during inflammation, an iNOS inhibitor abolished the competitive advantage of wild-type *E. coli* over the *moaA* mutant. Thus, these experiments have directly demonstrated the link between host inflammation, iNOS, RNS, and bacterial anaerobic respiration as a mechanism that can provide a growth advantage for Enterobacteriaceae in the GI tract during disease.

CF AND CHRONIC PULMONARY INFECTION BY *P. AERUGINOSA*

CF is a genetic disease where mutations of the CFTR gene lead to increased mucus production and chronic bacterial colonization in the lower airways [39, 40]. The respiratory epithelial cells are unable to properly secrete chloride into the airway surface liquid, resulting in dehydrated airway secretions that cause increased mucus viscosity and decreased mucociliary clearance [41, 42]. Because of the increased availability of nutrient-rich mucus and dysregulation of mucociliary clearance, bacterial counts markedly increase (≥10,000-fold), and a complex microbiota develops

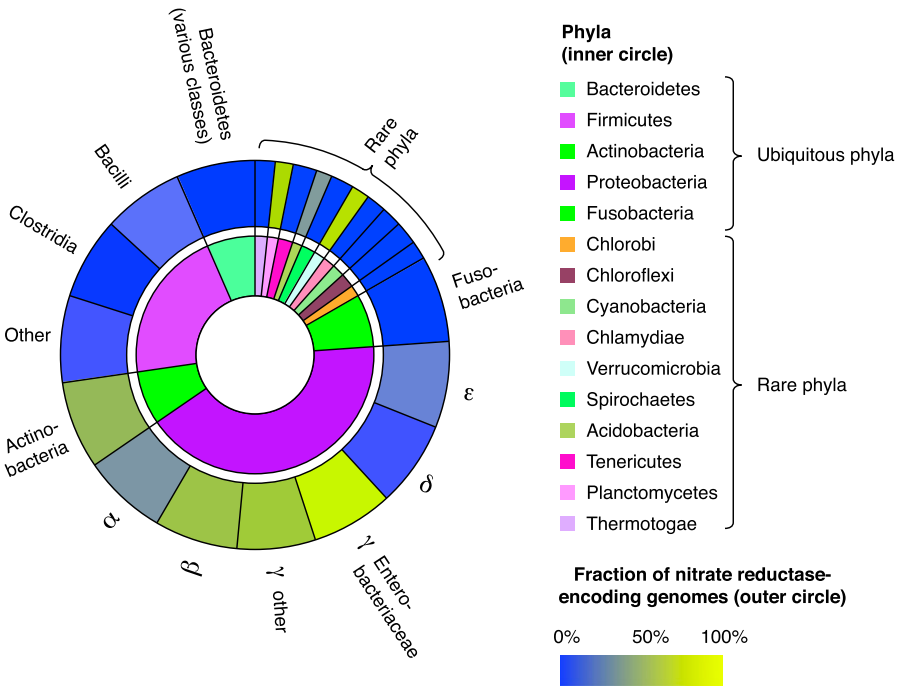

Figure 1. NO_3^- reductase capability within major phyla. NO_3^- reductase activity (E.C.1.7.99.4) was screened in 2476 genomes deposited within the KEGG (release 67.1). Prevalence of NO_3^- reductase ability was calculated as the fraction of genomes containing NO_3^- reductase genes per total genomes within each phyla (outer ring). Ubiquitous phyla are those commonly present in the gut microbiota, whereas rare phyla are those less commonly found in the gut microbiota (inner ring). Sector size is not proportional to the relative abundance of the phyla. Figure first published and reprinted here with permission from Winter *et al.* [3].

in the CF lung [40]. As the disease progresses and worsens, the microbial community decreases in diversity until 1 dominant microbe prevails; in most cases, this dominant organism is *P. aeruginosa* [43]. Once a chronic *P. aeruginosa* infection is established, it cannot be cleared by antibiotic treatment and typically persists until the end of the patient's life [44].

ANAEROBIC RESPIRATION IN *P. AERUGINOSA*

P. aeruginosa has long been held to be an obligate aerobic bacterium; however, recent studies have highlighted that this is not true. *P. aeruginosa* has a highly branched respiratory system for growth under both microaerophilic and anaerobic conditions. Experimental evidence suggests that *P. aeruginosa* prefers growth under microaerophilic conditions. In simulated growth experiments, *P. aeruginosa* showed its highest growth rate at 1% partial pressure of oxygen when compared to 5, 10, and 50% partial pressure of oxygen [45]. *P. aeruginosa* has 2 terminal oxidases that assist in growth and help create physiologic microaerobic conditions: the high-affinity oxidase cytochromes Cbb3-1 and -3-2. The cbb_3 oxidase genes are constitutively expressed, even in aerobic conditions, which is an unusual feature of *P. aeruginosa* biology, as these oxidases are typically repressed in the presence of high environmental oxygen [46]. In addition, *P. aeruginosa* isolated from CF airways are known to overproduce the exopolysaccharide alginate, which physically restricts the diffusion of oxygen into the cell

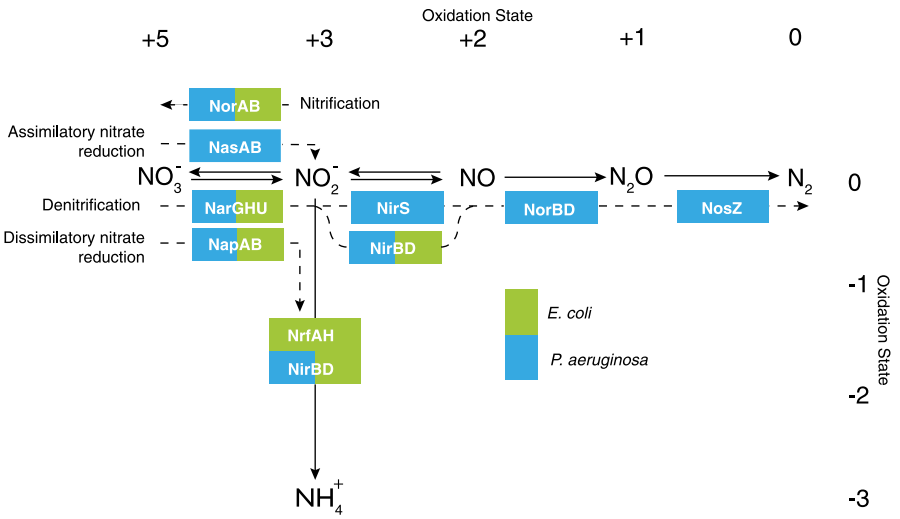

Figure 2. Nitrogen respiration pathways in *E. coli* and *P. aeruginosa*. The nitrogen reduction enzymatic pathways in commensal *E. coli* strain MG1655 and in *P. aeruginosa* strain PAO1. Each rectangle represents an enzyme, and the letters inside each rectangle represent the protein components of that enzyme. The rectangles are colored according to the presence of that enzyme in *E. coli* (light green) or *P. aeruginosa* (light turquoise). Solid arrows: possible directions in which the enzymatic reaction can occur; dotted arrows: actual direction of the indicated pathway. Diagram based on the information provided by KEGG.

and blocks the cell from oxidative stress [45, 46]. Low oxygen additionally triggers the production and release of important virulence factors, including elastase and pyocyanin [45]. Maintaining physiologic low-oxygen environment is likely an important aspect in *P. aeruginosa* colonization and pathogenesis.

When oxygen levels drop sufficiently, such that no molecular oxygen is available for aerobic respiration, *P. aeruginosa* is able to respire and grow through the denitrification pathway. Figure 2 displays the nitrogen reduction ability of *P. aeruginosa* alongside of *E. coli* and **Fig. 3** displays a more in-depth model of how we believe denitrification occurs at the cellular level in *P. aeruginosa*. In denitrification, nitrogen oxides are utilized as alternative terminal electron acceptors. Complete denitrification consists of 4 steps: (1) NO_3^- is reduced to NO_2^-; (2) NO_2^- is reduced to NO; (3) NO is reduced to N_2O and 4) N_2O is reduced to N_2. A reductase enzyme catalyzes each one of these steps. The genes required to produce the denitrification enzymes and proteins that regulate the anaerobic respiration system are arranged in 3 genomic clusters. NO_3^- reduction genes are found in the *narXL-narK1K2GHJI* (PA3871-PA3880) cluster; NO_2^- and NO reduction genes are arranged in the *nirSMCFDLGHJEN-norBCD* (PA0509-PA527.1) cluster and the N_2O to N_2 reduction genes are found in the *nosDFLRYZ* (PA3390-PA3396) cluster [46, 47]. Multiple steps in this pathway contribute to the generation of a proton motive force, primarily reduction of NO_3^- to NO_2^- by Nar and reduction of NO by cNOR [46] (Fig. 3). However, in order for nitrogen reduction to confer a growth advantage for *P. aeruginosa* there needs to be an abundance of available nitrogen in the CF lung.

CF, INFLAMMATION, AND *P. AERUGINOSA*

The substrates for denitrification can become available for anaerobic respiration through the production of RNS by inflammatory cells in mucus-rich regions of CF lungs [46, 48, 49]. The mucus within the CF lung is thick and mostly stagnant [48]. Upon infection, *P. aeruginosa* forms biofilms within the thick mucus, and these biofilms become surrounded by high densities of neutrophils that deplete the surrounding mucus of molecular oxygen through the production of ROS and RNS [50–52]. Direct *in situ* measurements of oxygen in CF sputum have confirmed that it ranges from microaerobic to anaerobic [53]. The presence of obligate anaerobes in the CF sputum and upregulated expression of the global anaerobic regulator Anr in *P. aeruginosa* isolates from CF-infected patients further demonstrate the lack of molecular oxygen availability in the CF lung [54, 55]. Analysis of the regulatory networks within *P. aeruginosa* reveals a complex system that actually limits the exposure of the bacterial cell to molecular oxygen (O_2), even under fully aerobic conditions. As discussed earlier, *P. aeruginosa* encodes a fully functional electron transport system that utilizes NO_3^- or NO_2^- as a terminal electron receptor for anaerobic respiration, via reduction of N_3 to N_2 ("denitrification"). The first step in this process is mediated by NO_3^- reductase, encoded by *narG*. Mutants mutated in the 2-component NO_3^- sensor-response regulator, and in membrane NarG display altered motility and biofilm formation compared to wild-type *P. aeruginosa* PAO1 [49].

We propose the model in **Fig. 4**; that analogous to the situation in the GI tract, inflammation in the CF lung likely provides the NO_3^- substrates for *P. aeruginosa* to grow via nitrogen respiration. The CF lung is chronically inflamed, irrespective of infection and lung disease. Multiple studies have found evidence of chronic inflammation in the CF lung, even in the absence of bacterial infection or the onset of lung disease [56–58]. Partly because of the chronic state of inflammation, the sputum within the CF lung contains sufficient nitrogen products to support growth of *P. aeruginosa* via anaerobic denitrification. Both neutrophils and alveolar macrophages found in CF sputum produce RNS and ROS [51, 59, 60]. Activated macrophages can release NO continuously for weeks, such that, during chronic inflammation, NO concentrations in the local environment can reach highly cytotoxic levels [59]. The continual release of NO from immune cells leads to increased levels of NO_3^- and NO_2^- in the CF sputum [61]. As stated previously, NO and O_2^- react to form $ONOO^-$, which is then catalyzed into NO_3^- and NO_2 [27]. For every mole of $ONOO^-$ created, ~0.7 mole of NO_3^- and 0.3 mole of NO_2^- is produced [27]. NO_3^- is also produced when NO is oxidized by the flavohemoglobin [62]. This process is necessary to protect *P. aeruginosa* from killing by NO from inflammatory cells [27]. In support of the frequency of the above reactions, exhaled NO from patients with CF is

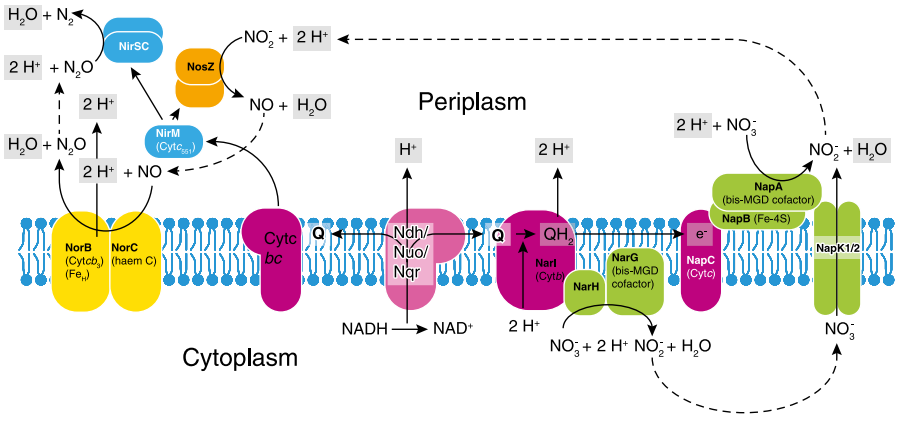

Figure 3. Proposed denitrification model in *P. aeruginosa*. Model built based on information provide in Williams *et al* [80]. Nar, respiratory NO_3^- reductase; Nap, periplasmic NO_3^- reductases; Nir, NO_2^- reductase; Nos,nitrous oxide reductase; Nor, nitric oxide reductase; NarK1/2, putative NO_3^-– NO_2^- antiporter; Cytc, cytochrome; Q, quinone; Nuo, NADH quinone oxidoreductase 1. Sizes of proteins are not to scale.

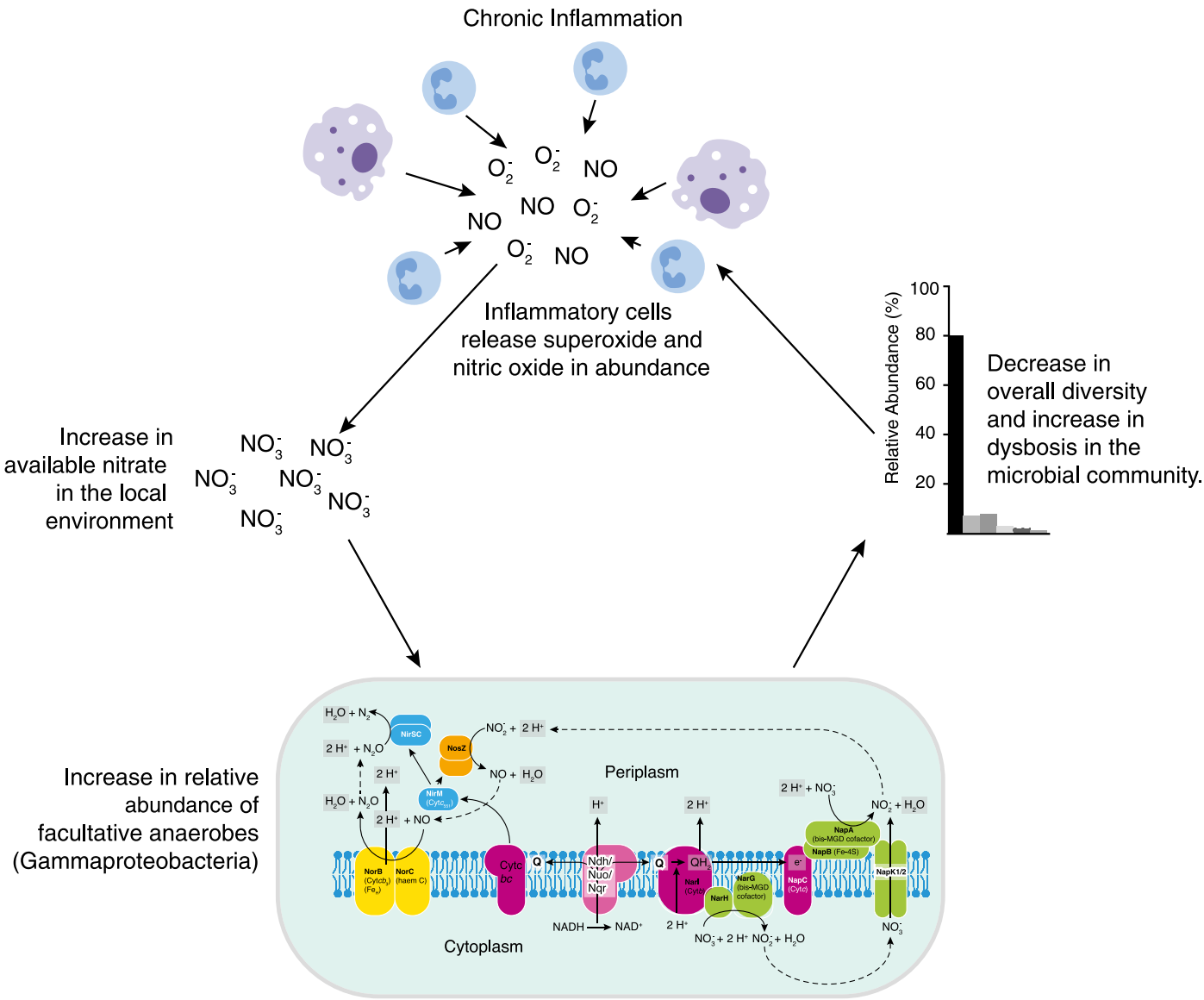

Figure 4. How inflammation affects the microbiota at a body site. Inflammatory cells release O_2^- and NO into the local environment. These are transformed into NO_3^-, which is then utilized by facultative anaerobes as a nutrient source. Facultative anaerobes, primarily Gammaproteobacteria, increase in relative abundance, leading to a decrease in overall diversity and dysbiosis in the microbial community. Dysbiosis in the microbiota can fuel the inflammatory state, leading to a feedback-loop–like state at the body site.

lower than that from non-CF individuals, whereas NO_3^- levels are considerably higher [61, 63–65]. The above reactions produce enough substrate for *P. aeruginosa* to utilize denitrification for growth [27]. *P. aeruginosa* increases in relative abundance, leading to a decrease in overall diversity and dysbiosis in the microbial community. Thus, one of the consequences of the upregulated host inflammatory response in patients with CF is the production of terminal electron acceptors for anaerobic respiration that allow *P. aeruginosa* to grow, persist, and outcompete all other microbial members of the CF lung microbial community.

Inflammation is a common feature of chronic respiratory diseases such as asthma and chronic obstructive pulmonary disease [2, 66, 67]. It has been observed in several studies that the relative proportion of Proteobacteria in the lungs increases during disease. Less is known about the relationship between inflammation and bacterial outgrowth in these diseases, compared to the state of the field in CF. However, it seems likely that comparable mechanisms are at play in these obstructive airway diseases.

COULD THERE BE INFLAMMATION/ BACTERIAL COLONIZATION FEEDBACK LOOPS?

Evidence suggests that Gammaproteobacteria not only benefit from the effects of inflammation, they also contain molecular components that directly enhance the inflammatory response. MAMPs on the surface of bacteria interact with receptors on

immune cells to initiate inflammation. One of the most potent MAMPs expressed by Gram-negative bacteria is LPS, which interacts with TLR4 on immune cells [68]. The lipid-A moiety of LPS is required for its endotoxic ability and is composed of a 1,4′-bis-phosphorylated diglucosamine backbone attached with 5 or 6 acyl chains. Two genes determine how LPS is acylated; bacteria with *LpxL* have LPS with 5 acyl (penta-acylated) chains and bacteria with both *LpxL* and *LpxM* have 6 acyl (hexa-acylated) chains. LPS that are penta-acylated are 100-fold less immunostimulatory when bound to TLR4 than LPS that is hexa-aceylated [69]. Although the *LpxL* gene is found in most Gram-negative bacteria, Gammaproteobacteria carry both the *LpxL* and *LpxM* genes and, therefore, have the more immunostimulatory version of LPS. The lungs of individuals with difficult asthma have up to a 7-fold increase of hexa-acylated LPS-producing bacteria than the lungs of healthy individuals, suggesting there is a link to inflammatory respiratory disease and the presence of more immunostimulatory LPS-producing bacteria [69]. In fact, the difference in immune induction between commensal *Prevotella* sp. and COPD/asthma-associated Proteobacteria is likely related to differences in LPS structure [68, 70]. In addition to LPS induction of TLR4, bacteria can also induce an immune response through a TLR9 interaction with unmethylated CpG motifs [71]. The optimal motif for inducing an immune response in humans is GTCGTT [72], and the optimal motif for activating mouse and rabbit immune cells is PuPuCGPyPy (Pu is purine and Py is pyrimidine) [73]. Certain members of the Gammaproteobacteria, including *P. aeruginosa*, have high frequency of both GTCGTT and PuPuCGPyPy motifs [74]. The high level of these immunostimulatory motifs would lead to a higher induction of inflammation when the bacteria are phagocytosed by immune cells. A feedback loop is thus envisioned where the same bacteria that contain the most immunostimulatory molecular components are the same bacteria that benefit most from an inflammatory environment.

Another potential feedback loop that occurs between bacterial colonization and respiratory inflammation occurs during exacerbations of respiratory diseases. Exacerbations are marked by rapid increases in inflammation, which lead to a worsening of disease symptoms and a reduction of respiratory function [75]. The increase in inflammation during exacerbations leads to an increase in inflammatory byproducts, such as iNOS released by neutrophils, and an increase in mucus production, which reduces the oxygen tension of the respiratory environment [53]. These changes in the lung environment alter the bacterial community by selecting for specific microbes. Paired specimen studies examining bacterial communities before and after exacerbations during chronic lung disease have found a community shift away from Bacteroidetes, toward an increase in Proteobacteria [76–78]. Proteobacteria contain multiple immunostimulatory molecular components (such as lipid-A moieties and CpG motifs) that further increase inflammation, leading for even more selective pressure and shift toward a Proteobacteria-dominated community [75]. The positive feedback loop that occurs in exacerbations is thus similar to the one proposed in Fig. 4, suggesting that respiratory inflammation, whether acute or chronic, follows a similar pattern where Proteobacteria harness the changes in environment due to inflammation to outcompete other members of the local microbiota.

CONCLUDING REMARKS

For bacterial communities, the environment of the gut and lung, like cities in a Dickens novel, are distinct but at the same time quite similar [1, 2, 4–6, 79]. The gut experiences high shear flow and nutrient availability; the healthy respiratory tract does not. The gut is nearly uniform in temperature across its length; the respiratory tract is not. The surface of the gut is lined with mucus; the distal respiratory tract is not, but is bathed instead in lipid-rich surfactant. The gut is largely anaerobic; the healthy respiratory tract is not. However, neither site supports anaerobic respiration, but this tale changes in the setting of chronic inflammation, in which the lung is flooded with mucus to create anaerobic zones and, in both the gut and lungs, dense concentrations of RNS that selectively favor the growth of facultative anaerobic Gammaproteobacteria. In short, host inflammation propels a convergence of the environmental growth conditions within these 2 organs, and bacterial growth in the diseased respiratory tract comes to resemble that in diseased colons. There is far, far more to uncover as it relates to inflammation and promotion of chronic bacterial growth in this emerging tale of two sites.

AUTHORSHIP

B.S.S., R.P.D., and G.B.H. all contributed to the written body of the review. B.S.S. created Figs. 2, 3 and 4.

ACKNOWLEDGMENTS

The work was supported by U.S. National Institutes of Health, National Heart, Lung, and Blood Institute Grants R01HL114447 (to G.B.H.) and T32HL00774921 (to B.S.S.) and funds from The Nesbitt Program for Cystic Fibrosis Research (to G.B.H.).

DISCLOSURES

The authors declare no conflicts of interest.

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KEY WORDS:
nitrate reduction · *E. coli* · *P. aeruginosa* · cystic fibrosis · inflammatory bowel disease