Genomic analysis of respiratory capacities in the human gut microbiome. By D.A. Ravcheev[1] and I. Thiele[1], [1]*Luxembourg Centre for Systems Biomedicine, University of Luxembourg, Luxembourg*

The human intestine is an organ with unique anatomy, physiology, and microflora. The features of the human gut create the steep oxygen gradients from stomach to rectum, and from intestinal wall submucosa to intestinal lumen (1). Such gradients, together with the presence of various products of food digestion, produce a rather large variety of ecological niches for the gut inhabiting microorganisms. Human gut microbial communities were intensively studied during last years (2-9), but, surprisingly, almost nothing is known about the respiratory capacities of the gut microbiota. Only in a small number of studies, mouse models were used for analysis of respiration in *Escherichia coli* (10, 11) or *Salmonella enterica* (12, 13). Nonetheless, systematic analysis of the respiratory capacities*, in silico* or i*n vivo*, has not been done yet.

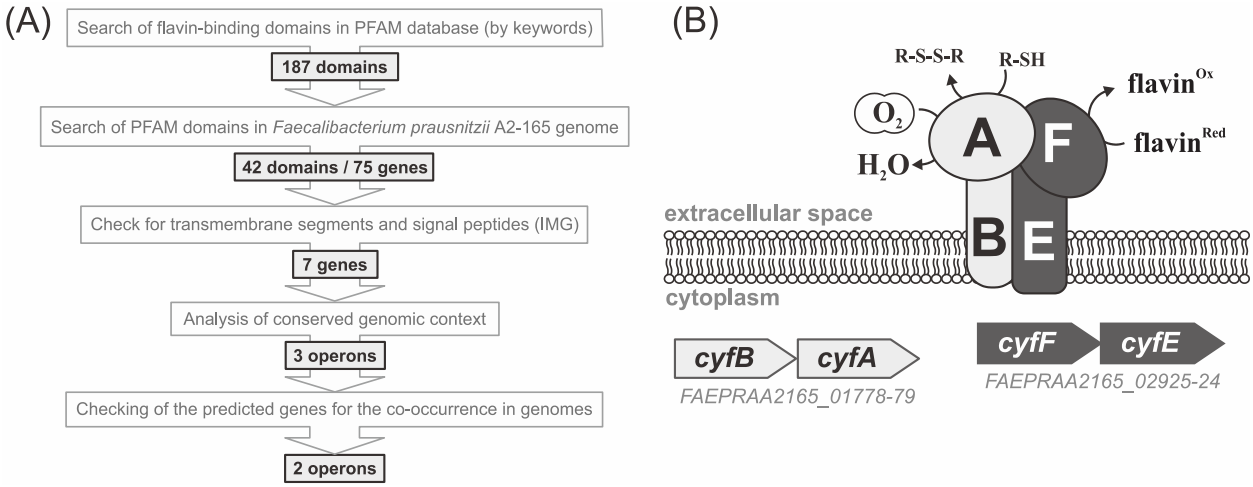
In this study (14), we aimed to investigate the respiratory capacities of the human gut microbiome. To identify respiratory genes in microbial genomes, we applied a set of comparative genomics and genome context-based techniques for accurate annotation of respiratory reductases. Based on the list of human gut microbes (15, 16), we selected 255 genomes of human intestine inhabitants available in the PubSEED (17) and IMG systems (18).

During the steeply dissipating oxygen gradient in the intestinal lumen, representatives of the human gut microbiome should demonstrate various capabilities to utilize molecular oxygen. To assess these different capabilities, we studied the distribution of aerobic reductases among the reference set of genomes. Two types of known aerobic reductases can be distinguished, (1) a high-affinity cytochrome *bd* reductases and (2) an aerobic reductases with various cytochromes that are characteristic for aerophilic organisms. Microaerobic reductases were found in 132 of the 255 reference genomes (51.8%), whereas aerophilic reductases were found in 29 of the 255 references genomes.

Additionally, in this work we predicted the novel microaerobic reductase. The gram-positive bacterium *Faecalibacterium prausnitzii* A2-165 has been shown to grow under microaerobic conditions in the presence of flavins and thiols while using an extracellular electron shuttle system (19, 20). Using the set of computational methods, based on the sequence and genome-context analysis (Fig. 1A), we predicted two operons that probably encodes proteins for this reductase. This reductase was called CyfABEF. The Cyf reductase probably contains two membrane proteins CyfB and CyfF, catalytic subinit CyfA, and flavin-binding subunit CyfF (Fig. 1B).

In order to define the repertoire of electron acceptors utilized by the reference set of genomes, we identified genes for all known aerobic reductases of electron acceptors (for references see Table S2), with the exception of reductases for metal and organohalide respiration. Overall, we looked at 32 types of anaerobic reductases and 26 of them were found in the reference genomes (Table S1). These enzymes are able to reduce tetrathionate, thiosulfate, polysulfide, sulfite, adenylyl sulfate, heterodisulfides, fumarate, trimethylamine N-oxide (TMAO), dimethyl sulfoxide (DMSO), nitrate, nitrate, nitric oxide, nitrous oxide, selenate, and arsenate. In the reference genomes, no genes have been found for the reduction of ethylbenzene, chlorate, and perchlorate.

Fig. 1. (A) The workflow for prediction of micoaerobic reductase Cyf in *F. prausnitzii*. (B) The model for structural organization of the reductase Cyf, locus tags are shown for genome of *Faecalibacterium prausnitzii* A2-165.



Together with the expansion of reductase specificities to homologous proteins, we also predicted one thiosulfate reductase, which has no known homologs with the same specificity. This reductase was found in genome of *Burkholderiales bacterium* 1_1_47, and was called by us Tsr. The Tsr reductase probably contains for the cytochrome *c-552/4* like protein and homolog of the nitrous oxide reductase) proteins. The orthologs of the Tsr operon were found in genomes of *Shewanella* spp., where these genes are induced by thiosulfate (21).

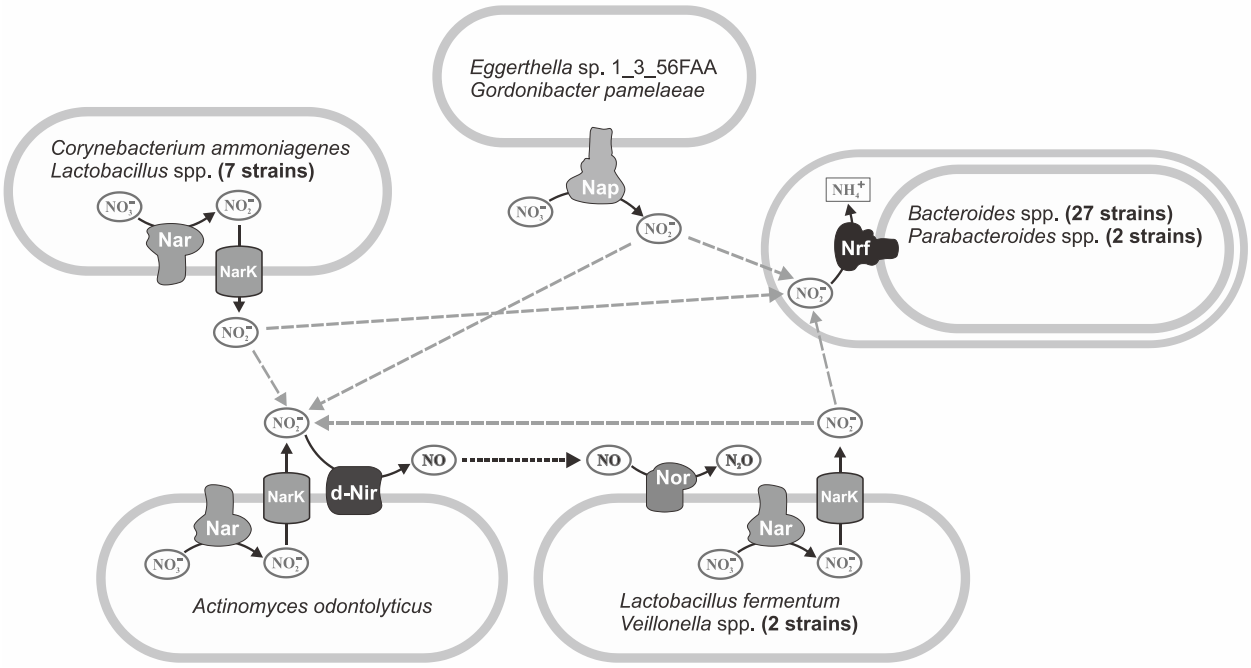
Based on the distribution of aerobic, microaerobic, and anaerobic reductases, we classified all reference genomes to aerophilic, microaerophilic and anaerobic. Among the 255 studied genomes, 29 (11%) were identified as aerophilic because of the presence of aerobic type reductases. Yet another 111 (44%) genomes were defined as microaerophilic, *e.g.*, having only microaerobic but not aerobic reductases. The residual 115 (45%) genomes were defined as anaerobic because of the absence of either microaerobic or aerobic reductases. Such distribution of the human gut inhabitant is in good agreement with data about oxygen concentrations found in the intestinal lumen.

The reference genomes demonstrated perceptible variations in the distribution of respiratory reductases. The most common respiratory enzyme is the microaerobic reductase Cyd found in 126 genomes (Table S1). Among anaerobic reductases, the fumarate reductase Frd was the most widespread one, as it was found in 93 of the studied genomes (36%). Other widespread respiratory reductases were nitrite reductase Nrf (found in 55 genomes, 22%), nitrate reductases Nar and Nap (found in 36 (15%) and 27 (11%) genomes, respectively), and DMSO/TMAO reductase Dms (found in 23 genomes, 9%). The other reductases were found in one to 19 studied genomes.

Various nitrogen oxides, such nitrate, nitrite, nitric oxide, and nitrous oxide often used as electron acceptors under anaerobic conditions (22-25). During the analysis of anaerobic respiration in the human gut reference genomes, we found that some studied genomes did not contain the full set of respiratory enzymes allowing the reduction of nitrate to ammonia or to

molecular nitrogen. The presence of such incomplete pathways can be explained by the hypothesis that microorganisms can could exchange byproducts of respiratory activity. For verification of this hypothesis, we analyzed all complete genomes for the presence for assimilatory reductases for nitrogen oxides and also for the presence of transport proteins required for respiratory and assimilatory reduction of these compounds. Pathways for the reduction of nitrogen oxides were found in 73 genomes. Only 28 genomes contain all enzymes required for the nitrate reduction to ammonia, and the denitrifying pathway was found only in one genome. In 11 genomes only nitrate but not nitrite reductases were detected. Otherwise, in other 29 genomes from genes for only nitrite reductase were found. Overall, 44 analyzed complete genomes have partial pathways for the reduction of nitrogen oxides. All these organisms are able to inhabit the same zone of the human intestine, so, there is no apparent barrier for metabolite exchange between them. Thus, we proposed that organisms with the partial pathways could exchange by nitrite and nitric oxide (Fig. 2).

Fig. 2. Predicted exchange pathways for nitrogen oxides in human gut microbe.



Conclusions. In this work, we first applied systematic analysis of the respiratory reductases in a reference set of 255 genomic sequences for human gut microbes. Such analysis of the large number of genomes is a necessary link between two 'traditional' approaches: study of model organisms and metagenome analysis. We analyzed the distribution of multiple aerobic, microaerobic, and anaerobic respiratory reductases, and also of energy conserving hydrogenases that under certain conditions can act as reductases of electron acceptors. In addition to the annotation of previously known enzymes, we also predicted one novel microaerobic reductase and one thiosulfate reductase. Based on the distribution of various reductases we divided reference genomes by ecological niches. Such division was in a good agreement with previous data about oxygen relations of these organisms and allowed us to improve previous predictions for phenotypes of a number of microorganisms. Additionally, based on the genomic analysis, we

reconstructed the network for the exchange of nitrogen oxides between various organisms inhabiting in the human gut. Extension of the presented work to respiratory dehydrogenases, their electron donors, and to the genes for the quinone biosynthesis will further improve our knowledge and understanding of the metabolism of human gut bacteria and their interactions to each other and to the human organism.

1. Espey MG. (2013) *Free Radic Biol Med* **55,** 130-40.
2. Eckburg PB, Bik EM, Bernstein CN*, et al.* (2005) *Science* **308,** 1635-8.
3. Flint HJ, Scott KP, Duncan SH*, et al.* (2012) *Gut Microbes* **3,** 289-306.
4. Gill SR, Pop M, Deboy RT*, et al.* (2006) *Science* **312,** 1355-9.
5. Kinross JM, Darzi AW, andNicholson JK. (2011) *Genome Med* **3,** 14.
6. Leimena MM, Ramiro-Garcia J, Davids M*, et al.* (2013) *BMC Genomics* **14,** 530.
7. Lozupone CA, Stombaugh JI, Gordon JI*, et al.* (2012) *Nature* **489,** 220-30.
8. Maurice CF, Haiser HJ, andTurnbaugh PJ. (2013) *Cell* **152,** 39-50.
9. Sonnenburg JL, Chen CT, andGordon JI. (2006) *PLoS Biol* **4,** e413.
10. Jones SA, Gibson T, Maltby RC*, et al.* (2011) *Infect Immun* **79,** 4218-26.
11. Spees AM, Wangdi T, Lopez CA*, et al.* (2013) *MBio* **4**.
12. Lopez CA, Winter SE, Rivera-Chavez F*, et al.* (2012) *MBio* **3**.
13. Winter SEandBaumler AJ. (2011) *Gut Microbes* **2,** 58-60.
14. Ravcheev DAandThiele I. (2014) *Environ Microbiol* [in press].
15. Nelson KE, Weinstock GM, Highlander SK*, et al.* (2010) *Science* **328,** 994-9.
16. Qin J, Li R, Raes J*, et al.* (2010) *Nature* **464,** 59-65.
17. Disz T, Akhter S, Cuevas D*, et al.* (2010) *BMC Bioinformatics* **11,** 319.
18. Markowitz VM, Chen IM, Palaniappan K*, et al.* (2014) *Nucleic Acids Res* **42,** D560-7.
19. Khan MT, Browne WR, van Dijl JM*, et al.* (2012) *Antioxid Redox Signal* **17,** 1433-40.
20. Khan MT, Duncan SH, Stams AJ*, et al.* (2012) *ISME J* **6,** 1578-85.
21. Beliaev AS, Klingeman DM, Klappenbach JA*, et al.* (2005) *J Bacteriol* **187,** 7138-45.
22. Ferguson SJ. (1998) *Curr Opin Chem Biol* **2,** 182-93.
23. Gonzalez PJ, Correia C, Moura I*, et al.* (2006) *J Inorg Biochem* **100,** 1015-23.
24. Kraft B, Strous M, andTegetmeyer HE. (2011) *J Biotechnol* **155,** 104-17.
25. Luque-Almagro VM, Gates AJ, Moreno-Vivian C*, et al.* (2011) *Biochem Soc Trans* **39,** 1838-43.