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25. BACTERIAL SPECIES AS PARTNERS AND PATHOGENS

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IS IT MORE TO BACTERIAL SEX THAN EXPLORING THE FITNESS LANDSCAPE OF OTHER ORGANISMS?

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SUMMARY

Horizontal gene transfer (HGT) has played and still plays an important role in adaptive evolution in bacteria. This is evident in the rapid emergence and spread of antibiotic resistance determinants worldwide as well as the abundance of horizontally acquired DNA in the ever-increasing amount of completely sequenced bacterial genomes. When the evolutionary benefits of HGT are discussed, many researchers forget that these observations of rare gene transfer events from close as well as distantly related bacteria describe the *effects* of these rare transfer events. In this report the different hypotheses that exist on the evolution of sexual reproduction in eukaryotes, one of the classical problems in evolutionary biology, is discussed in the context of bacterial evolution. I focus on competence for natural transformation, the only strictly bacterially encoded mechanism of HGT. It is argued that a plethora of mutually not exclusive mechanisms may account for the evolution and maintenance of natural transformation.

HORIZONTAL GENE TRANSFER; COINCIDENTAL EVOLUTION IN GIANT LEAPS

Through binary fission bacteria reproduce in an almost strictly asexual mode. The main sources of genetic diversity, the substrates for natural selection, are generated through the few mutations that escape the DNA repair machinery as well as through genomic deletions, inversions, and duplication/ amplification events. Even when acting in concert, these processes are relatively slow and limited by the genetic potential within a single cell. However, three para-sexual processes: conjugation, transduction, and natural transformation, allow bacteria to acquire genetic material from outside the cell and thereby engage in horizontal gene transfer (HGT), the prokaryotic version of sex. Through HGT bacteria can acquire adaptive genes, operons, and clusters of genes such as pathogenicity islands that already have passed through the trials and tribulation of natural selection even in other bacterial species. Thus, whereas bacteria are not as dependent on sexual reproduction as most eukaryotes they are not necessarily picky about their partners when environmental conditions favour inter-species "mating".

The effects of HGT are spectacular and these mechanisms of gene transfer play prominent roles in bacterial evolution particularly in habitat- and niche

expansions (*Levin* and *Bergstrom*, 2000; *Ochman* et al., 2000; *Thomas* and *Nielsen*, 2005). Despite the obvious advantage of HGT in niche adaptation and responses to abrupt environmental changes such as the immune system of vertebrates or antimicrobial agents, "exploration of the fitness landscape" (*Dubnau*, 1999) was not necessarily the selective force responsible for the evolution or the maintenance of this ability to acquire genes from without. It has been suggested that coincidental evolution plays and has played an important role in the evolution and maintenance of conjugation and transduction. This is due to the need of conjugative elements such as plasmids and conjugative transposons as well as phage for continuous transfer and infection of new hosts (*Levin*, 1987; *Redfield*, 2001). Whatever beneficial genes and traits these infectious elements carry in order to provide their hosts a selective advantage to ensure their own maintenance, these genes may coincidentally be incorporated into the hosts genome through the recombination system in the bacterial host.

Natural transformation is mechanistically closer to sexual reproduction in eukaryotes than both conjugation and transduction. The uptake of naked exogenous DNA from the environment is a complex process that requires concerted actions of many chromosomal genes encoded within the more than 40 bacterial species reported to have this ability so far (*Johnsborg* and *Håvarstein*, 2009). Since Griffith's pioneering experiment with un-encapsulated *Streptococcus pneumoniae* (*Griffith*, 1928) followed by Avery and co-workers' isolation of "active transforming substances", later shown to be DNA (*Avery* et al., 1944), the evolution and maintenance of natural transformation has been and still is a controversial topic. Broadly, the hypotheses and arguments can be divided into two groups. 1) Natural transformation has evolved and is maintained to generate diversity in evolving bacterial populations through shuffling of genes and mutations within and between populations (shuffling hypotheses). 2) The diversity generated following the uptake of exogenous DNA is a coincidental by-product by other selective forces (coincidental evolution hypotheses).

Here I review the available experimental evidence in both groups of hypotheses and argue that the forces responsible for the origin and evolution of natural transformation not necessarily are the same that maintains it. Moreover, natural transformation is a trait found in bacterial species across different taxa and most likely evolved more than once. It is thus highly unlikely that a single selective force maintains this fascinating mode of bacterial sex across different bacterial species.

THE EVOLUTION AND MAINTENANCE OF NATURAL TRANSFORMATION: THE "GENETIC SHUFFLING" HYPOTHESES

A high number of papers address intraspecies events of HGT where genes and mobile genetic elements traverse the species barriers with completely novel phenotypes as the final outcome. The rapid spread of antimicrobial resistance determinants primarily through conjugative mobile elements (*Thomas* and *Nielsen*, 2005; *Roberts* and *Mullany*, 2011), but also following natural transformation events (*Johnsborg* and *Håvarstein*, 2009), has become one

of the paradigms in this field. These spectacular examples of gene transfer are however rare, and they often depend on strong, even lethal selection pressures in order for the novel phenotype to rapidly ascend in populations. Moreover the accessory genes and elements often come with a "biological cost" and may be lost from the population in the absence of positive selection (*Johnsen* et al., 2009). It is unlikely that these HGT events are directly involved in the maintenance and evolution of natural transformation. In the context of the "shuffling" hypothesis, the selective forces acting on the evolution and maintenance of natural transformation works within the population, as pointed out by Baltrus and co-workers recently (*Baltrus* et al., 2008). Broadly, the active uptake of exogenous DNA followed by integration in the genome through either the recombination system or through unknown mechanisms (*Bryan* and *Swanson*, 2011) leads to either combining multiple beneficial adaptive mutations within one cell to reduce the competition between different adaptive mutations in individual cells, a process referred to as clonal interference (*Gerrish* and *Lenski*, 1998). Alternatively, natural transformation and recombination may lead to the effective removal of deleterious mutations from the genome by breaking up linkage disequilibrium thus potentially reduce the mutational load (*Otto* and *Lenormand*, 2002). Finally, it is possible that natural transformation provides a selective advantage for the competent cell by an interplay between beneficial and deleterious mutations where beneficial mutations can "escape" from genomes where their selective effects are masked by other fitness reducing mutations, as indicated in fruit flies (*Rice* and *Chippindale*, 2001).

THE SHUFFLING HYPOTHESES AND THE EVIDENCE

Increased rates of adaptive evolution

According to the classical theory on the evolution and maintenance of meiotic sex recombination acts as a means to generate genetic diversity (i.e. novel genotypes) upon which natural selection may act. Broadly, recombination thus accelerates the adaptation rates to the environment. Levin and Cornejo recently reported data from a strictly theoretical study using mathematical modelling followed by computer simulations on the effects of mutation, recombination, selection, and inter-population competition in imaginary populations that could and could not engage in homologous recombination (*Levin* and *Cornejo*, 2009). Using recombination rates as reported from *E. coli*, *Streptococcus pneumoniae, Bacillus subtilis,* and *Haemophilus influenzae* conditions where recombination accelerated the adaptability of bacterial populations were identified. Their results suggested that when recombining populations were present in high densities they would eventually outcompete any non-competent competitor even when these had initially a slightly higher fitness. This is an important parameter to include since in general genes and proteins involved in DNA uptake as well as recombination most likely come with a cost, as shown for *B. subtilis* (*Johnsen* et al., 2009). Most interestingly *Levin* and *Cornejo* (2009) demonstrated that when the frequencies of competent competitors were low compared to the non-competent ones they would not invade even when they had a potential fitness advantage. This is most likely attributed to the fact that

recombination rates are density dependent (i. e. recombination rates are lower when donor-, and recipient numbers are low). These results thus strongly suggest that the selective forces responsible for the origin of recombination and ultimately natural transformation are not the same as the ones that maintains it, as previously suggested elsewhere (*Lenski*, 1999). Whereas addressing the "origin question" is a difficult task, it is equally important and interesting to understand the maintenance of natural transformation in bacterial populations.

To date four different reports have addressed the diversity hypothesis experimentally. *E. coli* was the model organism in two of these papers (*Souza* et al., 1997; *Cooper*, 2007) whereas the naturally transformable *Acinetobacter baylyi*, and *Helicobacter pylori* were used in the other two (*Bacher* et al., 2007, *Baltrus* et al., 2008). They all used experimental evolution set-ups where differences in adaptation rates were measured between strains and populations that were competent and non-competent (*Bacher* et al., 2007, *Baltrus* et al., 2008), recombination proficient/deficient and interactions with high and low mutation rates (*Cooper*, 2007), or the effect of immigrant DNA in the evolving populations was measured (*Souza* et al., 1997).

Cooper was the first to report increased adaptation rates directly related to the effects of recombination in bacteria (*Cooper*, 2007). The experimental design was clever in that he designed recombination proficient strains of *E. coli* by introducing plasmid F. These isolates (*rec*+) would recombine following F-mediated conjugal chromosomal transfer between members of the evolving population. The adaptation rates were measured relative to isogenic strains (*rec*-) that were made recombination deficient by deleting *traD*, a F-plasmid gene necessary for conjugal transfer. Cooper also tested the effect of recombination and increased mutation rates on these evolving *E. coli* populations by deleting *mutS*. Following 1000 generations of adaptive evolution the *rec*+ *mutS* genotypes adapted faster to the experimental conditions. When compared to *rec*- *mutS* and *rec*- populations only, it was clear that recombination was the main contributing factor to the increased adaptation rates. Cooper also followed the population dynamics of a previously described adaptive mutation in the regulatory gene *spoT* (*Cooper* et al., 2003) in the same strain. The results revealed that the *spoT* mutation also emerged in these evolving populations and ascended to fixation quicker in the recombination proficient-mutator populations than in the recombination deficient ones. Cooper further measured the fitness effects early and late in the selective sweeps of these mutations relative to contemporary clones and the ancestor. The results from late in the selective sweeps revealed that for *rec*- clones harbouring the *spoT* mutation fitness decreased relative to contemporary clones. The relative fitness between these *spoT* mutants and the ancestor did however not decrease, suggesting the presence of other beneficial mutations in the competing populations. Taken together the presented results suggest that recombination accelerated the adaptation rates and that this was due to reduced competition between different beneficial mutations in different hosts (i.e. clonal interference). These results may seem to contradict Souza, Turner and Lenski's 1997 report where the same problem was addressed using *E. coli* and almost identical experimental settings (*Souza* et al., 1997). These authors failed to show increased adaptation rates when recombination was added to the asex-

ual *E. coli* system through plasmid F-mediated high frequency of recombination (Hfr). The two reports however differed in the source of donor DNA. These authors added another *E. coli* strain (K12) that could not replicate in the experimental conditions, but transfer of chromosomal DNA easily occurred. The experimental set-up thus simulated inter-population HGT events. The results revealed increased genetic diversity in the "sexual" populations, scored by presence/absence of nine physiological loci, but the adaptation rates did not increase relative to the "asexual" populations. On first consideration these results seem to contrast Cooper's findings (*Cooper*, 2007). But they really don't, they underscore an important difference between the effects of recombination. Souza and coworkers really showed that adaptation rates did not increase in a constant environment when the populations imported genes from without. Moreover, when compared to Cooper, using the same *E. coli* B strain as Souza and coworkers, it is evident that the major difference was that in these experiments the evolving populations were allowed to shuffle mutations between individual cells in the same population (intra-population HGT events). The increased mutation rates in Cooper's experiments just optimized the selective effect of reduced clonal interference in the "sexual" adapting *E. coli* lineages.

E. coli is not naturally transformable, so how does these results fare when natural transformation is considered? Studies on recombination in *E. coli* could very well be relevant for populations were F-like plasmids are present in high frequencies. Nevertheless, the above-mentioned reports address the effects of recombination through highly optimized conjugation/recombination events and not natural transformation. It is clear that the average benefit of natural transformation must overcome the costs of the active DNA uptake and DNA processing machineries. Moreover, natural isolates of bacteria competent for natural transformation display varying transformation frequencies in the laboratory (*Cohan* et al., 1991). One interpretation of these results is that the ability to take up and integrate DNA is slowly lost from the populations investigated. Alternatively, it is simply the induction of competence that varies between different environments.

The first attempt to address the potential evolutionary benefit of natural transformation was done with the highly competent soil dweller *Acinetobacter baylyi* ADP1 (*Bacher* et al., 2007). Bacher and co-workers adapted wild type *com*+ and mutant *com*- populations deficient in DNA uptake (*ΔcomFEBC*::Kan^r-*sacB*) for 1000 generations under optimal growth and competence conditions. The authors then competed evolved populations against the ancestral clone in high and low-density experimental set ups. The results revealed faster adaptation rates for *com*+ under high-density competitions, but in the low-density competitions no differences were detected. The authors concluded that competence for transformation gave no consistent evolutionary advantage under the laboratory conditions used (*Bacher* et al., 2007). It should however be noted that none of the competitions were performed under the exact same conditions under which selection operated in these experiments. Moreover the serial transfer experiments were performed with strong bottlenecks close to the frequency of transformation. Therefore, unless new phenotypes generated by transformation displayed extraordinary increments in fitness, they would most likely be lost in the bottlenecks.

The first experimental evidence in favour of the "shuffling-hypothesis" in a naturally competent bacterium was provided from Baltrus and co-workers (*Baltrus* et al., 2008). As Bacher and co-workers did (*Bacher* et al., 2007), these authors tested the hypothesis that natural competence for transformation increases the adaptation rates in the naturally transformable *Helicobacter pylori*. In this report Baltrus and co-workers constructed a pair of *com+/com- H. pylori* (*ΔcomH*::Kan^r-*sacB*) strains and founded five replicate populations of each genotype. These 10 populations were then propagated in a serial transfer experiment that lasted for approximately 1000 generations. The experimental design varied from *Bacher* et al. (2007) in that the serial transfers were diluted 1:50 introducing a less pronounced bottleneck. At the end of the serial transfer experiment it was clear that the *com+* wt populations on average had 15% increased fitness relative to the com- populations when both were compared to the ancestral strains. The authors provided no experimental evidence for the evolutionary mechanism responsible for the increased adaptation rates, but argued that reduced clonal interference was the most plausible explanation (*Baltrus* et al., 2008).

Removal of deleterious mutations from the genome

Partly based on Kimura and Maruyama's work on mutational load Alexey S. Kondrasov proposed the *deterministic mutation hypothesis* in the mid 1980s; see *Kondrashov* (1988) and references therein. This hypothesis postulates that sex evolved and is maintained in large populations as a means to remove deleterious mutations from the genome. Whereas Kondrasov mainly argued in terms of eukaryotes this could very well hold for natural transformation in bacteria as well. The hypothesis has two important prerequisites; it absolutely requires that the added effect of detrimental mutations in terms of host fitness must be greater than each mutation alone, also called positive epistasis. Moreover it requires a mutation rate of one per genome per generation (*Kondrashov*, 1988). The imaginary scenario in transformable bacterial populations would effectively be removal of the deleterious mutations by homologous recombination with donor DNA harbouring the wt allele. This can also be seen as a means to retain genomic integrity.

Elena and Lenski set out to test the central prediction of positive epistasis in the deterministic mutation hypothesis in their model organism *Escherichia coli* (*Elena* and *Lenski*, 1997). They started out with a strain that already had adapted to the experimental conditions for 10,000 generations. This strain was from Lenski's now famous experimental evolution project that in 2011 reached over 50,000 generations in continuous serial transfer cultures (http://myxo.css.msu.edu/). A total of 225 strains with 1, 2, and 3 randomly inserted mini-Tn*10* transposons (75 in each group) were constructed. Fitness measurements relative to the unmutated genotype were conducted in the same growth medium as to where the strain was already adapted (*Elena* and *Lenski*, 1997). The mean relative fitness data for each mutational group (i.e. 1, 2, and 3 mini-Tn10 insertions) was plotted as a function of number of insertions (mutations). Linear regression analyses subjected to conservative statistical analyses revealed a linear relationship between the fitness effects and number of insertions. These data were clearly inconsistent with positive epistatic interactions between deleterious mutations where a negative non-linear relationship would be in support

of the hypothesis. In a separate experiment Elena and Lenski provided an explanation for this particular linear fitness function. Different combinations of mini-Tn*10* insertions showed both antagonistic and synergistic effects on fitness when again compared to the mutation-free strain. The authors concluded that "the mutational deterministic hypothesis seems to fail not because interactions between deleterious mutations are very rare, but rather because synergistic and antagonistic interactions are both common." (*Elena* and *Lenski*, 1997).

In 1964 Muller proposed a hypothesis called "Mullers' ratchet". The "ratchet" symbolizes irreversible reduction in fitness in finite asexual populations due to the accumulation of deleterious mutations. The effect is particularly pronounced in small or bottlenecked populations were genetic drift acts in concert with the ratchet and randomly removes "the least mutated" genotype most often representing genomes with higher fitness. According to this hypothesis sexual recombination may slow down or even stop the ratchet through the removal of detrimental mutations (*Muller*, 1964; *Felsenstein*, 1974)

Through an experimental regime where single colonies of *Salmonella typhimurium* LT2 were streaked onto agar plates repeatedly for some 1700 generations Andersson and Hughes tested if fitness in these populations would decay when compared to the founding clone (*Andersson* and *Hughes*, 1996). In roughly 1% of the lineages (5/444) a reduced growth rate were observed but perhaps most importantly not a single mutant with increased fitness was isolated. These data are indeed compelling evidence for accumulation of deleterious mutations in *Salmonella typhimurium*, the key prediction of Mullers ratchet. Similar evidence was presented by Moran following sequence analyses of coding genes in the endosymbiotic *Buchnera spp* (*Moran*, 1996). These bacteria fit both expectations of the ratchet hypothesis in that their populations are extremely small, and they undergo severe bottlenecks when their host cells divide. Consistent with Muller's ratchet Moran's analyses revealed accumulations of slightly deleterious mutations.

In conclusion, the reports reviewed above show that conditions, where shuffling genes and mutations within bacterial populations occur, clearly exists. The relevance with respect to the evolution and maintenance of natural transformation is however limited because *E. coli*, *Salmonella* and *Buchnera* are not competent for natural transformation.

Elena and Lenski's data are rather convincing in that the absolute requirement of positive epistasis between deleterious mutations in the deterministic mutation hypothesis is not met in *E. coli* (*Kondrashov*, 1988, *Elena* and *Lenski*, 1997). It would be interesting to see if similar results could be obtained in species competent for natural transformation. The hypothesis should however be tested in different competent species as to test the generality of such results. There is another caveat about the deterministic mutation hypothesis in the context of natural transformation. Incoming DNA from neighbouring cells may very well originate from dead cells, and it is reasonable to assume that these DNAs have detrimental mutations as well. Redfield, Schrag, and Dean showed, using mathematical models, that this constitutes a potential cost for naturally transformable bacteria (*Redfield* et al., 1997).

The Muller's ratchet hypothesis clearly has some support for the general requirement of irreversible accumulation of deleterious mutations

(*Andersson* and *Hughes*, 1996; *Moran*, 1996). However, to the best of my knowledge, no experiments have yet demonstrated that natural transformation, and/or recombination removes deleterious mutations from the genome and subsequently gives the competent strain a selective advantage as would be anticipated from these highly interesting results.

THE COINCIDENTAL EVOLUTION HYPOTHESES

The experiments reported by Cooper and by Baltrus and co-workers (*Cooper*, 2007; *Baltrus*, et al., 2008) clearly suggest that when certain environmental conditions are met, recombination may increase the adaptation rates to novel and relatively static environments. However several other hypotheses exist that address the evolution and maintenance of natural competence for transformation. Non of these hypotheses are mutually exclusive, and in fact, it is likely that they may act in concert with the ”shuffling-hypotheses” in order to maintain the competence machinery in naturally transformable bacteria. These hypotheses have a common theme; the acquisition of exogenous DNA and potential integration in the bacterial genome is a coincidental by-product of the induced competence machinery. The selective forces responsible for its maintenance are however acting on the levels of DNA repair and DNA uptake for nutritional purposes.

The DNA repair hypothesis

The hypothesis of *Felsenstein* (1974) postulates that recombination is maintained in populations as a by-product of DNA repair. The “repair” hypothesis has gained support from some very prominent evolutionary biologists, including John Maynard Smith. For an excellent review see: *Bernstein* et al. (1987). On the evolution and maintenance of natural competence for transformation in bacteria this hypothesis is indeed compelling. If DNA taken up by natural transformation is used as templates for recombinational repair this would provide a stable positive selective pressure for the maintenance of the complex competence machinery almost from generation to generation. Michod and co-workers tested this hypothesis in a series of reports on the naturally transformable *Bacillus subtilis* (*Michod* et al., 1988; *Wojciechowski* et al., 1989; *Hoelzer* and *Michod*, 1991). In these reports the survival of transformants as well as transformation rates were tested following increasing doses of ultraviolet irradiation (UV) either when DNA was added to the cultures before irradiation (DNA-UV) or after (UV-DNA). This experimental set-up allowed the authors to isolate the effect of competence on survival because DNA was added as a substrate for natural transformation after UV-irradiation in the UV-DNA treatments. In the first report the results showed that in the UV-DNA treatments transformants survived better on average than the total cells in the population. The reverse was true in the DNA-UV experiments. The reported transformation rates were also higher in the UV-DNA than in the DNA-UV treatments (*Michod* et al., 1988). In the second report from these authors further support in favour of the repair-hypothesis was provided in experiments where homologous and heterologous (plasmid) DNA was the substrate for transformation. Increased transformation rates following UV-treatment were only observed when

homologous DNA was added to *B. subtilis* cultures. In the same study, a set of DNA repair-, and recombination deficient mutants (*uvrA, and recA*) displayed the expected increased susceptibility to UV irradiation as well as increased transformation rates. Finally, gene-expression levels measured through *lacZ* fusions of DNA damage-induced genes (*din*) suggested that DNA damage induced the SOS response in both competent and non-competent members of the population (*Wojciechowski* et al., 1989). In the last report Hoelzer and Michod showed that wether the donor DNA came from cells that was UV-irradiated or not the transformants survived still better than the non-competent members of the population (*Hoelzer* and *Michod*, 1991).

Taken together these data suggest that competent *B. subtilis* cells, when exposed to a DNA damaging agent, survive better than non-competent members of the same population. The experimental setup where DNA was added before and after UV treatments as well as the demonstrated lack of effect on survival from plasmid mediated transformants suggested strongly that the observed results were due to the DNA uptake, and/or the transformation process including induction of the competence machinery in *B. subtilis*. This is indeed interesting, but the effect of exogenous DNA used as templates in DNA repair in *B. subtilis* is not assured. As pointed out in the authors' own discussion, the growth arrest observed in competent *B. subtilis* cells (Nester and Stocker, 1963) could also play a role in these experiments. It could be hypothesized that this allows for more efficient DNA repair before growth is resumed and that this affects fitness of competent cells. A few years after Michod and co-worker's publications it was clear that competence for transformation in *B. subtilis* is dependent on, and is regulated by the *comK* gene product (*van Sinderen* et al., 1995). ComK is a master regulator turning on more than 100 genes including those necessary for DNA uptake and recombination (*Berka* et al., 2002). Dubnau and co-workers termed this "state" the K-state and argued that this is a "unique adaptation to stress" in *B. subtilis* (*Berka* et al., 2002). Upon dilution in fresh medium, the ~10% competent members of the *B. subtilis* population do not rapidly resume growth as the non-competent cells do. ComGA blocks replication and cell division for approximately 2 hours and this "checkpoint" is present until ComK is degraded and is likely to ensure DNA repair as a consequence of recombined DNA and/or followed by the competence induction (*Haijema* et al., 2001). The effects ComK and ComGA are independent of the presence of DNA. From these later findings it is clear that Michod and co-workers lacked an important control in their experiments. If the increased transformant-survival observed in the UV-DNA experiments would be independent of DNA, this would effectively provide evidence against the repair hypothesis. Other reports in *B. subtilis* and *Haemophilus influenza* also provide evidence that do not favour the repair hypothesis. Mongold performed experiments like Michod and co-workers did in *B. subtilis* in *H. influenza* (*Mongold*, 1992). The initial results were in support of the repair hypothesis in that increased survival was observed in UV treated cultures following the addition of genomic homologous DNA. However, when Monogold added a 9 kb *H. influenza* DNA fragment this small piece of DNA effectively increased survival as well. These data suggested that the added DNA did not repair UV-induced damages by recombinational repair. If natural competence for transformation

evolved as a means to repair double stranded DNA breaks one could argue that DNA damage should induce competence for transformation. Redfield showed that DNA damaging agents added to *B. subtilis* and *H. influenza* cultures (UV, and mitomycin C) did not induce competence in the strains tested (*Redfield*, 1993). However, in two other naturally competent as well as clinically relevant bacterial species *Streptococcus pneumonia*, and *Helicobacter pylori*, natural competence is induced following exposure to DNA damaging agents such as mitomycin C and fluoroquinolones (*Prudhomme* et al., 2006; *Dorer* et al., 2010). Interestingly, none of these organisms have an intact SOS response and Prudhomme et al argued that competence development in *S. pneumonia* plays the role of SOS induction in *E. coli* (*Prudhomme* et al., 2006). In *H. pylori* Dorer, Fero and Salama proposed a "positive feedback of DNA on DNA damage responsive genes" where upon DNA damage competence is induced (*Dorer* et al., 2010). According to the proposed model, RecA induce increased expression of both a lysozyme-like protein that stimulates donation of DNA from neighbouring *H. pylori* cells as well as a type four-secretion system (T4SS) that increases the import of exogenous DNA (*Dorer* et al., 2010). Recently, it was also demonstrated that competence is induced in *Legionella pneumophilia* after exposure to genotoxic stress (UV-irradiation, mitomycinC, and fluoroquinolons) (*Charpentier* et al., 2011). None of these excellent reports addressed the evolutionary forces responsible for the maintenance of these complex DNA uptake machineries. Taken together, the presented data from *S. pneumoniae*, and *H. pylori* provides new information that may favour the DNA repair hypothesis on the maintenance and possibly the origin of natural competence for transformation in these species. It is also clear that the hypothesis is testable in carefully planned laboratory experiments, but to date no conclusive experimental evidence in favour of the DNA repair hypothesis has been provided.

The nutrient hypothesis

DNA can be used as a nutrient, either as a source of phosphate, nitrogen, or carbon, or alternatively as a source of nucleotides for the synthesis of nucleic acids (*Stewart* and *Carlson*, 1986; *Redfield*, 2001). The ability to exogenously acquire genes "for breakfast" (*Redfield*, 1993) or the less mundane synthesis of nucleic acids could provide an evolutionary advantage since nucleotide synthesis is catabolically very expensive, from the perspective of a bacterial cell (*Stewart* and *Carlson*, 1986; *Redfield*, 2001). Several arguments have been launched in favour of the nutrition hypothesis. Naturally transformable bacteria are often dwelling in environments where extracellular DNA is abundant such as in mucosal layers of the respiratory, and gastrointestinal tracts as well as in soil (see: *Redfield*, 2001 and references therein). Natural competence for transformation is induced in some naturally transformable species when the nucleotide pool is drained (*H. influenza*) (*MacFadyen* et al., 2001) upon transfer from a rich to a nutrient limited medium (*B. subtilis*) (*Dubnau*, 1999), or competence peaks in late log/early stationary phase (*Acinetobacter baylyi*) reviewed in (*Redfield*, 2001). These observations are all consistent with an organism tuned in on DNA uptake when resources are depleted. It should however be noted that evidence in *A. baylyi* is not as clear-cut as stated by Redfield (*Redfield*, 2001). *A. baylyi* reaches maximum competence after diluting an overnight culture in fresh medium

(*Palmen* et al., 1994). If the exceptionally high transformation frequencies obtained in this organism is due to an abrupt up-regulation of the competence machinery following the sudden availability of nutrients, or if competence developed in late log-phase is not yet clear. *S. pneumoniae* develops competence rapidly in most members of the population for a brief period in mid-log phase (*Johnsborg* and *Håvarstein*, 2009). Also disfavouring the nutrient hypothesis is the apparent excess of induced genes induced in the competent state as well as the uptake specificity seen in some species (for example *H. influenza*, and *Neisseria gonorrhoeae*) (*Dubnau*, 1999).

The nutrient hypothesis for the evolution and maintenance of natural competence for transformation is still controversial. It should however be testable in the laboratory, and at present main problem of the repair hypothesis is the lack of experimental evidence, more than lack of good arguments.

THE EPISODIC SELECTION HYPOTHESIS

We recently proposed a novel hypothesis for the maintenance of competence and natural transformation in *B. subtilis,* episodic selection (*Johnsen* et al., 2009). Based on Nester and Stocker's early observations that competent *B. subtilis* are refractory to penicillins (i.e. they do not grow for a few hours upon transfer to fresh medium) (*Nester* and *Stocker*, 1963) we hypothesized, and provided theoretical as well as experimental evidence for, that the growth arrested sub-population has an competitive advantage when exposed to conditions where growing cells (i.e. non-competent *B. subtilis*) are killed. Our experimental data showed that competence deficient mutants (*comK::kan*) had a 15% increased fitness when compared to the competent wt *in vitro*. This is evident from the fact that not only does ~10% of the *B. subtilis* population not grow for a couple of hours when exposed to fresh medium, but these cells also express the additional genes induced by the K-state regulator ComK (*Haijema* et al., 2001; *Berka* et al., 2002). As a consequence, in the absence of a more frequent selective pressure than the rare acquisition of beneficial genes and mutations a competence deficient mutant would ascend in the population and competence would rapidly be lost.

We also demonstrated experimentally the other central theoretical predictions.

1) The competence deficient mutant was more susceptible to penicillin G than the wild type in time-kill experiments.

2) In direct competitions between the wt and the competence deficient mutant the initial 15% cost of inducing competence was mitigated and even highly beneficial in experiments where the mixed populations were treated with pulses of penicillin G.

3) When pulses of penicillin G were not sufficient to maintain the wt in mixed competition experiments the uptake of an antibiotic resistance determinant (chloramphenicol resistance) from the competing competence deficient mutant rapidly fixed in the wt population following the addition of chloramphenicol to the competitions (*Johnsen* et al., 2009).

The episodic selection hypothesis suggests that two forces act synergistically to maintain competence for the uptake and integration of exogenous DNA. First, when environmental

stressors are present that kills off growing cells (i.e. non-competent *B. subtilis*) more rapidly than the competent members of the population competence will be selected for. If these episodes are frequent competence provides a direct selective advantage. If not so frequent, these episodes "buys time" for episodes of the second type: When conditions favour the uptake of beneficial DNA (operons, genes, and mutations) competence and natural transformation may provide just that DNA, and competence would provide a clear fitness advantage. Recently, Wylie and co-workers added several layers of complexity in a purely theoretical study on bacterial competence that included both the recombination as well as the persister (growth arrest) features in *B. subtilis* (*Wylie* et al., 2010). Their simulations included homologous recombination (genes and mutations from within the populations). During adaptive evolution, recombination and persistence were indirectly favoured and disfavoured, respectively. However, when a single cell had the ability to stochastically switch between competence and vegetative growth, as shown experimentally for *B. subtilis* (*Maamar* et al., 2007), this was beneficial and cells with the ability to switch could invade a non-switching population. Wylie and co-worker's report differ from *Johnsen* et al. (2009) in that the growth arrest is an inherent, but detrimental effect of the DNA uptake process. Thus a trade-off exists between these two effects of competence development. In other words, growth arrest is a necessary burden to endure for the ability to take up DNA and possibly to gain beneficial mutations and genes that are positively selected for when these cells escape growth arrest. They also suggested that the actual switching was selected for, but for a different model with interpretations, see Libby and Rainey's report on bet hedging (*Libby* and *Rainey*, 2011)

CONCLUDING REMARKS

The evolution and maintenance of competence for natural transformation, the only solely bacterial encoded sexual mechanism, is still controversial. It is clear that theory and experiments have predicted and even demonstrated that under certain conditions recombination in bacteria is favoured evolutionarily by the ability to shuffle mutations *within* the population by reducing clonal interference. This is consistent with the classical explanation for the evolution of sex in eukaryotes. If this is true in naturally competent bacteria is not so clear. Evidence from experimental evolution set-ups is ambiguous, at best. A set of not mutually exclusive hypotheses also exists for the evolution of sex, also applicable to the evolution and maintenance of natural competence for transformation in bacteria. This problem is not only academically interesting it also has practical implications. An increased understanding of why bacteria exchange genes could very well give us clues as to curb the ever increasing emergence and spread of antibiotic resistance that are causing trouble in clinics and communities worldwide.

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BACTERIA AND HORMONES: WHY THE SCIENCE OF MICROBIAL ENDOCRINOLOGY MATTERS TO DISEASE AND WELL-BEING

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SUMMARY

Nearly 95% of the cells that constitute an individual are prokaryotic and reside in the gut. Additionally, bacteria are as well abundantly present on external surfaces such as the skin. The recognition that these microorganisms, whether commensal or pathogenic, can actively synthesize and respond to neuroendocrine hormones that had previously been viewed as belonging to eukaryotic systems, offers the potential to revolutionize our concept of not only disease causation, but also maintenance of homeostasis. This convergence of seemingly disparate fields, microbiology, endocrinology and neurophysiology, is the emerging translational medicine discipline known as microbial endocrinology and had at its beginnings the demonstration that elaboration of stress-related neuroendocrine hormones by the nervous system innervating the intestinal tract could be utilized as environmental signals by bacteria whether within the gut or on surfaces such as the skin to initiate growth and produce virulence factors as part of the infectious disease process. Although initially met with scepticism, this interdisciplinary concept that a *direct* bacterial-neuroendocrine hormone interface (based on evolutionary inter-kingdom signalling) could contribute to infectious disease pathogenesis has now begun to shape medical thought regarding infectious disease and maintenance of general health.

INTRODUCTION

The ability of stress to alter the pathogenesis of infection has been documented in human and animal studies since the early 1900's (*Cohen* and *Williamson*, 1991; *Peterson* et al., 1991). Depending on the stressor employed and the infectious agent chosen for challenge, stress has been shown to either increase, decrease or not affect survival (*Peterson* et al., 1991). Since stress-induced activation of the neuroendocrine system has been amply documented to affect immune competence (*Bateman* et al., 1989), it has been generally concluded that the ability of stress to alter the pathogenesis of infection must be mediated by neuroendocrine-immune interactions. In the majority of publications the infectious organism is perceived to be somewhat of a bystander since any observed differences in infectivity due to stress are explained in terms of neuroendocrine suppression or enhancement of immune system function. That the infectious organism itself may be equally respond-

ing to the neuroendocrine outflow resulting from the stress event is usually not considered. Indeed, in the current conceptual framework of psychoneuroimmunology as a triad composed of stress, endocrine and immune components (*Ader* et al., 1995), the infectious agent is not generally included.

It is not the intent of this paper to obviate a role for stress-induced modulation of immunity in the pathogenesis of infection. The collective data demonstrating neuroendocrine mediation of immune responsiveness is overwhelming (*Ader* et al., 1995; *Bateman* et al., 1989; *Cohen* and *Williamson*, 1991; *Peterson* et al., 1991). Indeed, the development of the present microbial endocrinology theory regarding direct, non-immune, interactions between the neuroendocrine system and bacteria in the pathogenesis of infectious disease is a direct outgrowth from work in my laboratory on the ability of social conflict stress to modulate immune responsiveness (*Lyte* et al., 1990a, 1990b, 1991). The results from these studies have demonstrated that the immune compartments most affected by social conflict stress are those concerned with first-line protection against infectious challenge, notably the mononuclear phagocyte system. A dramatic increase in the phagocytic ability of splenic and lung macrophages was observed in stressed mice as compared to handled and home cage controls (*Lyte* et al., 1990a, 1990b). From these results it was expected that susceptibility to infection should be decreased in stressed mice since components of the first line defence against infection were enhanced by the stress of social conflict. Surprisingly, this was not found to be the case (*Lyte*, 2010a). Stressed mice were more susceptible to infection with the rapid proliferation of bacteria occurring within hours upon entrance into the social conflict stressed, but not handled control, host (*Lyte*, 2010a).

These seemingly contradictory results led to the consideration that microorganisms may be equally capable of responding to the neuroendocrine outflow resulting from the stress event as compared to immune cells (*Lyte*, 1993). Could direct effects of hormones on the pathogenicity of the infectious microorganism provide as equally compelling an explanation for stress induced-alterations of infectivity as that currently obtained with the effects of stress on immune cell function? This question essentially represented the beginning of the microbial endocrinology theoretical framework (*Lyte*, 2010a).

RELEVANCE OF MICROBIAL ENDOCRINOLOGY FOR UNDERSTANDING THE ROLE OF STRESS IN INFECTIOUS DISEASE

The range of hormones and the variety of microorganisms in which they have been identified is very large (*Lenard*, 1992). The presence of insulin in microorganisms has been the most extensively documented with its biological activity demonstrated in every microorganism examined to date (*Lenard*, 1992). Other hormones isolated from microorganisms which have been demonstrated to show biological activity in mammalian cells include corticotropin from *Tetrahymena pyriformis* (*LeRoith* et al., 1982), somatostatin from *Bacillus subtilis* (*LeRoith* et al., 1985) and *Plasmodium falciparum* (*Pan* et al., 1987) and progesterone from *Trichophyton mentagrophytes* (*Schar* et al., 1986). Numerous other hormones identified by radioimmunoassay and chromatographic behaviour, as well as the presence of the corre-

sponding putative receptor, have also been demonstrated in various microorganisms. A recent comprehensive analysis by Roshchina (*Roshchina*, 2010) of the wide spectrum of neurohormones and related cognate receptors that have been isolated form microorganisms highlights the presence in microorganisms of what are otherwise thought to be more commonly associated with mammalian systems (*Roshchina*, 2010).

Investigators have debated the significance of such hormones in microorganisms for decades. The most widely accepted theory concerns the use of such hormones as a form of intercellular communication (*Dohler*, 1986; *Lenard*, 1992; *LeRoith* et al., 1986). Indeed, recent studies have shown that the growth of colonies of *Escherichia coli* involves a high degree of specialization of function by individual bacteria (*Shapiro* and *Hsu*, 1989) and presumably the need for some form of intercellular communication to accomplish this goal. This concept of microbial hormones serving in some capacity as a primitive vertebrate nervous system can further be expanded to the realm of infectious disease in man (*Lyte*, 2010b). According to a microbial endocrinology-based approach, microorganisms entering into a stressed host could utilize the hormones produced during the stress event as environmental cues by which they would sense their surroundings. The development of pathogenicity would then, in part, depend on the ability of the particular microorganism to respond to the type of hormonal environment that it encounters upon entrance into the host.

EVOLUTIONARY CONSIDERATIONS IN MICROBIAL ENDOCRINOLOGY

The evolution of microorganisms preceded that of vertebrates such as man. It is perhaps somewhat surprising to learn that the presence of what are thought to be almost exclusively vertebrate neurotransmitters are in fact widely dispersed throughout nature. For example, in addition to its presence in vertebrates, norepinephrine has been additionally identified in plants (*Smith*, 1971), insects (*Pitman*, 1971), and fish (*Guerrero* et al., 1990). The ubiquitous distribution of norepinephrine throughout nature suggests that microorganisms in general have had ample time preceding the evolution of man to come into contact with a wide spectrum of hormones and develop mechanisms by which to synthesize as well as recognize hormones.

The widespread presence of neurohormones in the environment and in the microorganisms themselves therefore suggests that recognition of mammalian hormones might serve as a type of environmental signal by which microorganisms may sense their surroundings and initiate pathogenic processes. Considering that the gastrointestinal tract has abundant neuronal innervation with a high amount of elaborated neurochemicals such as the stress-related catecholamine dopamine (*Eisenhofer* et al., 1997) and that the majority of infections are acquired via the per oral route, it would seem therefore reasonable to suggest that the hormonal environment that a microorganism encounters upon entrance into the host may play a role in determining susceptibility to infection.

The possibility has been suggested that many of the mammalian cell-to-cell signalling systems that involve

small rapidly diffusible molecules such as the compounds that comprise communication within the neuroendocrine system arose from horizontal gene transfer from bacteria (*Iyer* et al., 2004). As such, it should not be surprising to learn that the complete biosynthetic pathway for catecholamines that is well known in eukaryotic systems is also present in bacteria (*Iyer* et al., 2004).

NEUROPHYSIOLOGICAL ALTERATIONS ACCOMPANYING INFECTION

Numerous reports have documented that dramatic elevations in the levels of plasma catecholamines occur during the course of infection with Gram-negative bacteria (*Benedict* and *Grahame-Smith*, 1978; *Groves* et al., 1973; *Jones* et al., 1988). Gruchow (*Gruchow*, 1979) observed that elevated levels of stress-related catecholamine activity as measured by 3-hydroxy-4-methoxy mandelic acid excretion preceded the development of acute infectious disease episodes. Groves and colleagues (*Groves* et al., 1973) observed that the levels of norepinephrine and epinephrine were significantly higher in postoperative patients that developed severe septic conditions than in patients with uncomplicated postoperative recovery. Benedict and Grahame-Smith reported that although plasma norepinephrine levels were elevated in patients with septic shock, these levels did not directly correspond with alterations in blood pressure or heart rate as would have been expected (*Benedict* and *Grahame-Smith*, 1978). Animal studies have also documented that several-fold elevations in catecholamines occur as a consequence of infection. For example, following challenge of conscious rats with live *E. coli* survival was negatively correlated with increasing levels of norepinephrine (*Jones* et al., 1988).

The origin of the increased peripheral sympathetic outflow during infection is complex and not yet well understood. Both adrenal and postganglionic sympathetic sources are believed to contribute to the overall rise in the catecholamines with the adrenal medulla contributing the major portion of epinephrine. The proportion of norepinephrine contributed by the adrenal medulla versus the amount released by peripheral sympathetic nerve endings is not certain since the majority of reports have solely examined plasma catecholamine levels. Complicating the understanding of the relative contribution of each to the overall increase in norepinephrine has been the fact that animal models employing live infection have often yielded different results than that employing endotoxin administration. Further, it should be remembered that although the majority of human and animal studies have documented the levels of catecholamines present during sepsis by determination of plasma concentrations, these levels reflect a spillover phenomenon and as such grossly underestimate the amount of catecholamines that may be present within an organ (*Kopin* et al., 1984; *Kovarik* et al., 1987).

Considering, then, that within the microenvironment of the tissue adequate levels of neurohormones may be available to an infectious microorganism upon entrance into the host could the pathogenesis of the resulting infection somehow related to the neurophysiological response of the host (*Lyte*, 1993)? Can the infectious agent

actively utilize the hormonal products of the host's neurophysiological response to stress, such as the elaboration of norepinephrine, to its own advantage?

HISTORICAL EVIDENCE SUGGESTING A ROLE FOR MICROBIAL ENDOCRINOLOGY IN INFECTIOUS DISEASE

The ability of neuroendocrine hormones to influence the *in vivo* growth of pathogenic bacteria was first observed in 1930 (*Renaud* and *Miget*, 1930). Prior to the advent of disposable syringes, metal needles and glass syringes were reused constantly between patients with only a cursory cleaning in alcohol. Patient to patient transmission of infectious disease was frequently encountered due to the inadequate alcohol treatment of syringe needles, which could only marginally kill actively growing (vegetative) bacterial cells, but not bacterial spores. As is well understood today, certain vegetative bacteria such as *Clostridium perfringens*, the causative agent of gas gangrene, can undergo sporulation. Such spores, which are formed from the vegetative cells under conditions of nutritional deprivation, are totally resistant to alcohol treatment and can only be killed by autoclaving.

Starting in 1930 reports associating the use of contaminated needles with administration of epinephrine solutions in the development of rapidly disseminating infections began to appear (*Brocard*, 1940; *Cooper*, 1946; *Renaud* and *Miget*, 1930). A previously used syringe needle to treat a gas gangrene patient was then used to administer epinephrine to a patient for urticaria (*Renaud* and *Miget*, 1930). Within 6 hours a fatal fulminating gas gangrene infection developed. These reports noting the rapidity of infectious spread in patients receiving epinephrine injections with contaminated needles led A.A. Miles in 1948 to begin a series of experiments examining the role of catecholamines in bacterial pathogenesis (*Evans* et al., 1948). In these experiments, the ability of epinephrine to modulate the *in vivo* growth of both Gram-positive and Gram-negative bacteria in a guinea pig model was conclusively demonstrated in tissue slices with enhancement of growth of bacteria co-injected with epinephrine that was log orders greater than that for control slices co-injected with saline (*Evans* et al., 1948). The authors concluded that the ability of epinephrine to dramatically enhance bacterial growth was due to some protective coating of the bacteria by epinephrine or an epinephrine-induced inhibition of immune cell function. The testing of each of these possible mechanisms, however, met with failure (*Evans* et al., 1948). Significantly, at no time did these authors or others suggest that the action of epinephrine on bacterial growth was due to a direct, non-immune effect as is currently proposed (*Lyte*, 1993, 2010a).

Interestingly, one of the frequently used techniques by microbiologists to enable gas gangrene infections to "take" in mice has been the co-injection of epinephrine along with *C. perfringens* (*Traub* et al., 1991). It can reasonably be inferred that this practice dates back to the decades old reports described above. It should also be appreciated that much of the historical evidence that has been proposed as the basis for microbial endocrinology rests in reports that have shown over the last 70 years that the intersection of bacteria with endocrinology, and for that

matter neurophysiology, can provide unexpected results. For example, it is well known that the emergence of the worldwide market for anxiety reducing drugs such as diazepam owe their development directly to the first muscle relaxant mephenesin (*Berger*, 1969). What is probably less well recognized is that mephenesin was originally developed as an antibiotic for use against Gram-negative bacteria during World War II and only through serendipity was it observed to cause muscle relaxation in mice (*Berger*, 1969).

RECENT EVIDENCE TO SUPPORT A ROLE FOR MICROBIAL ENDOCRINOLOGY IN INFECTION

The possibility that an infectious organism may respond to such neuroendocrine signals should not seem that unlikely. Work starting in the early 1990's demonstrated that the catecholamines can profoundly influence the *in vitro* growth of Gram-negative bacteria (*Lyte* and *Ernst*, 1992, 1993). Norepinephrine, in particular, was shown to increase the growth of members of the *Enterobacteriaceae* and *Pseudomonadaceae* families. Importantly, this effect of catecholamines on bacterial growth was shown to non-nutritional in nature. If the effect was simply a nutritional one, then epinephrine which has one more carbon atom than norepinephrine and hence can serve as a better energy source should have at least, if not better, ability to modulate bacterial growth when compared to norepinephrine. The results, however, conclusively demonstrated that an order of potency for the effect of the various catecholamines on growth existed for some of the Gram-negative bacteria such as *Y. enterocolitica* responding only to norepinephrine (*Lyte* and *Ernst*, 1992). Studies involving the use of α and ß adrenergic agonists and antagonists, as well as the less physiologically active enantiomer of norepinephrine, (+)-norepinephrine, suggested that a non-α, non-ß adrenergic receptor mediated process may play a role in norepinephrine-induced growth of Gram-negative bacteria (*Lyte* and Ernst, 1993).

More recent studies by a number of groups have confirmed and extended these early reports of direct stimulation of microbial growth by stress-related neurochemicals in both *in vitro* as well as *in vivo* systems (*Bearson* and *Bearson*, 2008; *Burton* et al., 2002; *Freestone* et al., 2000; *Kinney* et al., 2000; *Lyte*, 2004; *Oneal* et al., 2008; *Pullinger* et al., 2010a, 2010b; *Rahman* et al., 2000; *Reissbrodt* et al., 2002; *Sperandio* et al., 2003; *Vlisidou* et al., 2004). However, it should be noted that not all biogenic amines have similar effects on bacterial growth. For example, norepinephrine and dopamine, but not the indoleamine serotonin, increased the growth of a number of Gram-negative enteric pathogens (*Lyte* and *Ernst*, 1992; *Lyte*, 1997). The ability of stress-related biogenic amines to influence virulence-related properties of bacteria was also observed in the enterotoxigenic *E. coli* B44 strain where the production of the K99 pilus adhesin, which is involved in attachment and penetration of the bacterium into the intestinal mucosa, was shown to be increased in the presence of norepinephrine (*Lyte* et al., 1997).

Other reports have also noted the possible interaction between the host's endocrine environment and the proliferation of microorganisms. Intracellular steroid binding proteins in yeasts such as estradiol in *Coccidioides immitis* has led to the suggestion that mammalian

hormones may influence the proliferation of fungal infections especially in pregnant women (*Powell* et al., 1983). Interestingly, the host neuroendocrine environment may have opposite effects regarding the proliferation of a microorganism. For example, binding of the ß-adrenergic receptor on the surface of the protozoa *Trypanosoma cruzi* leads to an increase in cAMP levels and a subsequent inhibition in its rate of proliferation and differentiation (*de Castro* et al., 1987). As mentioned previously, insulin has been observed in every microorganism examined to date (*Lenard*, 1992). In patients with pre-existing diabetes mellitus, the pathogenesis of the rodent-borne infectious disease melioidosis has been shown to be dependent on serum insulin levels. Binding studies involving the causative agent of melioidosis, *Pseudomonas pseudomallei*, have demonstrated the presence in the bacterium of a specific, high affinity binding site for insulin (*Woods* et al., 1993).

Taken as a whole, these reports strongly suggest that an infectious organism may respond to a wide range of host neuroendocrine signals in an effort to establish a productive infection in the face of a competent immune system. The direct effect of neurochemicals on infectious agent replication may provide as equally compelling an explanation for stress-induced alterations of infectivity as that currently obtained with the effects of stress on immune cell function. Therefore, the consideration of the infectious process from a microbial endocrinological perspective may provide new insights into the mechanisms by which stress can alter host susceptibility to infectious challenge.

MICROBIAL ENDOCRINOLOGY AND INFECTIONS OF INDWELLING MEDICAL DEVICES

One of the foremost applications of microbiology endocrinology to infectious disease is in the intensive care clinical setting. The ability of bacteria to colonize indwelling medical devices, such as central venous catheters (CVCs), is recognized as the most common infection encountered in the intensive care setting (*Rello* et al., 1994). The incidence of nosocomial infections has been estimated at approximately 2 million cases per year with approximately half of those being associated with indwelling medical devices such as CVCs (*Darouiche*, 2004). The majority of catheter-associated nosocomial infections are caused by coagulase-negative staphylococci (C-NS), the normal skin commensal *S. epidermidis* being responsible for 50-70% of reported cases (*Rupp* and *Archer*, 1994).

In an effort to combat CVC-related infections and the possible subsequent progression to catheter-related bloodstream infection (CRBSI) (*Crnich* and *Maki*, 2001), the use of antimicrobial coated catheters has been proposed and evaluated in a number of clinical studies (*Crnich* and *Maki*, 2002, 2004; *Darouiche* et al., 1999; *Geffers* et al., 2003; *Marciante* et al., 2003; *McConnell* et al., 2003). However, concerns about the efficacy of antimicrobial-coated CVCs in the prevention of CRBSI remains (*McConnell* et al., 2003) as well as does their possible contribution to the emergence of drug-resistant clones (*Sampath* et al., 2001; *Tambe* et al., 2001). The environmental factors that may influence *S. epidermidis* adherence and biofilm formation are largely unknown (*Costerton* et al.,

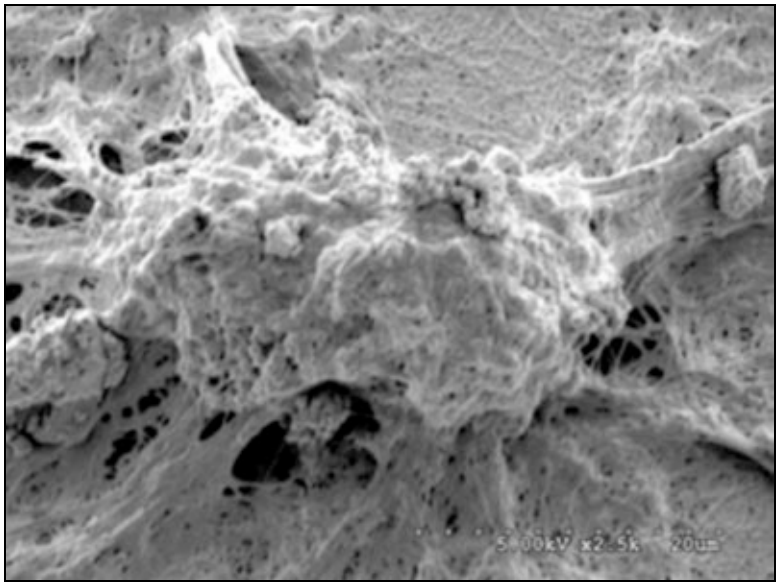

Figure 1: *S. epidermidis* biofilm formation in plasma-supplemented medium in the presence of the stress-related catecholamine norepinephrine. Little, or no, detectable biofilm formation was observed for the strain incubated in plasma-supplemented medium in the absence of catecholamines (control) cultures (not shown).

1999; *Watnick* and *Kolter*, 2000). Factors extend from disinfectants at the bedside (*Knobloch* et al., 2002) to the therapeutic drugs infused through catheter lines (*Lyte* et al., 2003). The examination of this latter environmental factor (*Lyte* et al., 2003) led to the application of the microbial endocrinology hypothesis to the study of the mechanisms governing bacterial colonization of indwelling medical devices. Specifically, could the drugs which are given to critically-ill patients for the maintenance of cardiac and renal function, namely the catecholamine inotropes, themselves be contributing to the ability of *S. epidermidis* to establish biofilms on CVCs as well as to the development of antimicrobial resistance and CRBSI.

Work from my laboratory has shown that catecholamine inotropic drugs may indeed serve through microbial endocrinology-based mechanisms as an aetiological factor in the bacterial colonization of indwelling medical devices due to their ability to stimulate *S. epidermidis* growth and biofilm formation (*Lyte* et al., 2003; *Neal* et al., 2001). The exposure of *S. epidermidis* to pharmacologically relevant concentrations of the widely used inotropic drugs, dobutamine and norepinephrine, resulted in increased biofilm growth and production of exopolysaccharide (the cellular excreted "glue" that holds cells in a biofilm together) as shown by both scanning electron microscopy and immunofluorescence (*Lyte* et al., 2003) and Figure 1. This demonstration of microbial endocrinology-based interactions in the development of medical important infections in patients receiving indwelling medical devices has led to the call for the design of new inotropic drugs that do not stimulate bacterial growth (*Singer*, 2007). Most recent has been the extension of these results in indwelling medical devices to the application of microbial endocrinology

to the design of new treatment modalities for even greater serious medical conditions such as post injury systemic immune response syndrome (*Lyte*, 2009).

CONCLUSION

There is growing recognition that inter-kingdom signalling, such as that exemplified by neuroendocrine hormones that are shared by both prokaryotic and eukaryotic cells, represents an interdisciplinary approach to understanding the biological processes that influence the pathogenesis of infectious disease as well as homeostasis. The recognition of this interdisciplinary field, which has been termed microbial endocrinology, represents the intersection between microbiology and neurobiology. The relevance of microbial endocrinology to health and infectious disease is increasingly being recognized by the medical community (*Everest*, 2007; *Singer*, 2007; *Stewart*, 2003) as well as the publication of the first dedicated to this sub-discipline within microbiology (*Lyte* and *Freestone*, 2010).

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INTESTINAL *LACTOBACILLUS REUTERI*: PARTNERS AND BENEFICIAL MICROBES

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INTRODUCTION

According to the Food and Agricultural Organization of the United Nations and the World Health Organization, probiotics are defined as "living microorganisms, which when administered in adequate amounts confer a health benefit on its host" (*FAO/WHO*, 2001). Elie Metchnikoff, who was best known as a laureate for Nobel Prize in Medicine in 1908 for his ground-breaking research in phagocytosis, was one of the first prominent scientists to introduce the concept of probiotics to the general public. He published a seminal report on association between longevity of Bulgarians and their consumption of fermented milk products (*Metchnikoff* and *Mitchell*, 1907). This observation suggested that ingestion of certain microbes could be beneficial for human health. Since then, probiotics had been widely marketed and consumed, mostly as dietary supplements or functional foods without proper validation of their promised beneficial effects. Significant advancements in probiotic research have occurred during the past two decades. Novel beneficial organisms have been identified and characterized. Mechanisms of probiosis include manipulation of intestinal microbial communities, suppression of pathogen, immunomodulation, stimulation of epithelial cell proliferation and differentiation and fortification of the intestinal barrier (Figure 1) (*Thomas* and *Versalovic*, 2010).

The Gram-positive bacterium *Lactobacillus reuteri* is a heterofermentative symbiont indigenous to the gastrointestinal tract of humans and many other vertebrates such as pigs, mice, and rats (*Walter* et al., 2010). A recent evolutionary genomic study revealed a molecular basis of host specificity among *L. reuteri* species, which may be due to physiological and immunological differences between different vertebrates (*Frese* et al., 2011). This species is generally regarded as safe and has never been shown to cause disease in humans (*Britton* and *Versalovic*, 2008). Results from basic science research and clinical trials have demonstrated potential beneficial effects of *L. reuteri* on human health, both in preventive and therapeutic aspects. This review will focus on how *L. reuteri* could affect the physiological processes of the host through intestinal immunomodulation, development and maintenance of the intestinal epithelium, and prevention or treatment of intestinal injury.

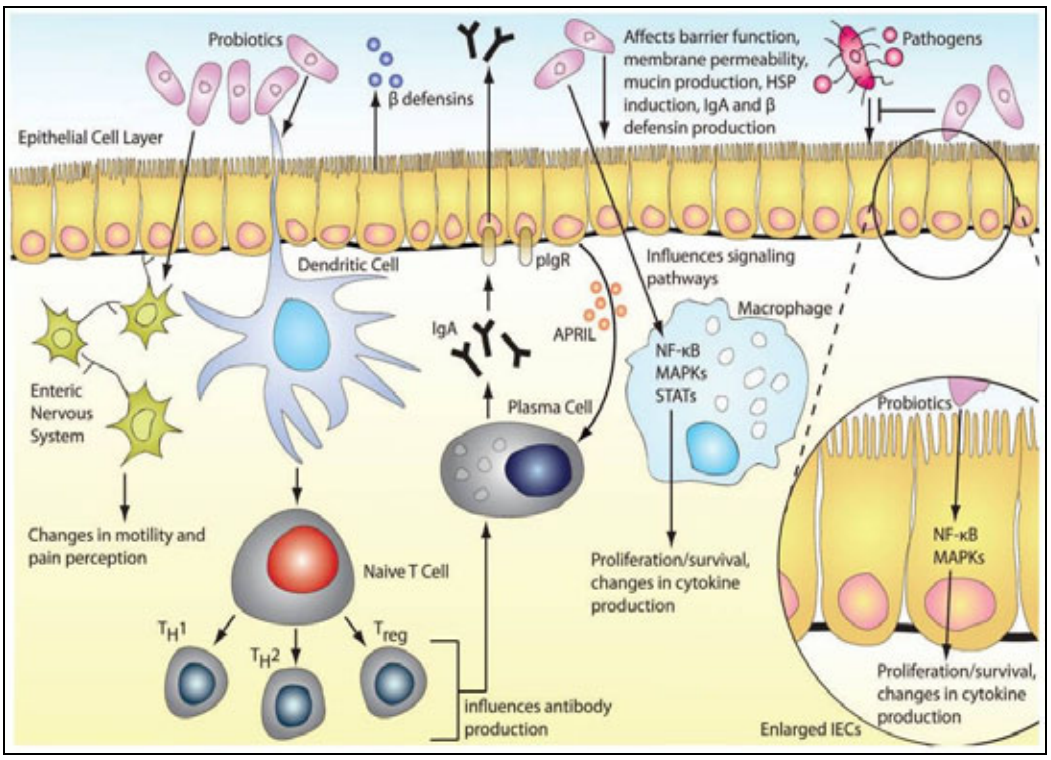

Figure 1: Mechanisms of probiosis in the human gastrointestinal tract. Probiotics affect intestinal functions in several ways. They may manipulate intestinal microbial communities and suppress growth of pathogens by inducing β-defensin and IgA production. Probiotics also enhance the integrity of intestinal barrier by maintaining tight junctions and inducing mucin production. Probiotics can modulate the immune system by mediating cytokine secretion through signaling pathways such as NFκB and MAPKs, which can also affect proliferation and differentiation of immune cells (such as T-cells) or epithelial cells. Moreover, changes in gut motility and pain perception can be altered through modulation of pain receptor expression and secretion of potential neurotransmitter molecules. APRIL, a proliferation-inducing ligand; hsp, heat shock protein; IEC, intestinal epithelial cell; Ig, immunoglobulin; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor-kappaB; pIgR, polymeric immunoglobulin receptor; STAT, signal transducers and activator of transcription; Treg, T regulatory cell. Figure reproduced from *Thomas* and *Versalovic*, 2010.

LACTOBACILLUS REUTERI AND INTESTINAL IMMUNOMODULATION

The human gastrointestinal tract contains approximately 10^{14} commensal bacteria (*Ley* et al., 2006). The intestinal immune system must be able to protect the host from pathogenic microbes, while still maintaining immunological hyporesponsiveness to members of the intestinal microbiome. Disruption of gut homeostasis may result in diseases associated with intestinal inflammation, such as inflammatory bowel disease (IBD), infections and colorectal cancer (*Artis*, 2008; *Karin* et al., 2006).

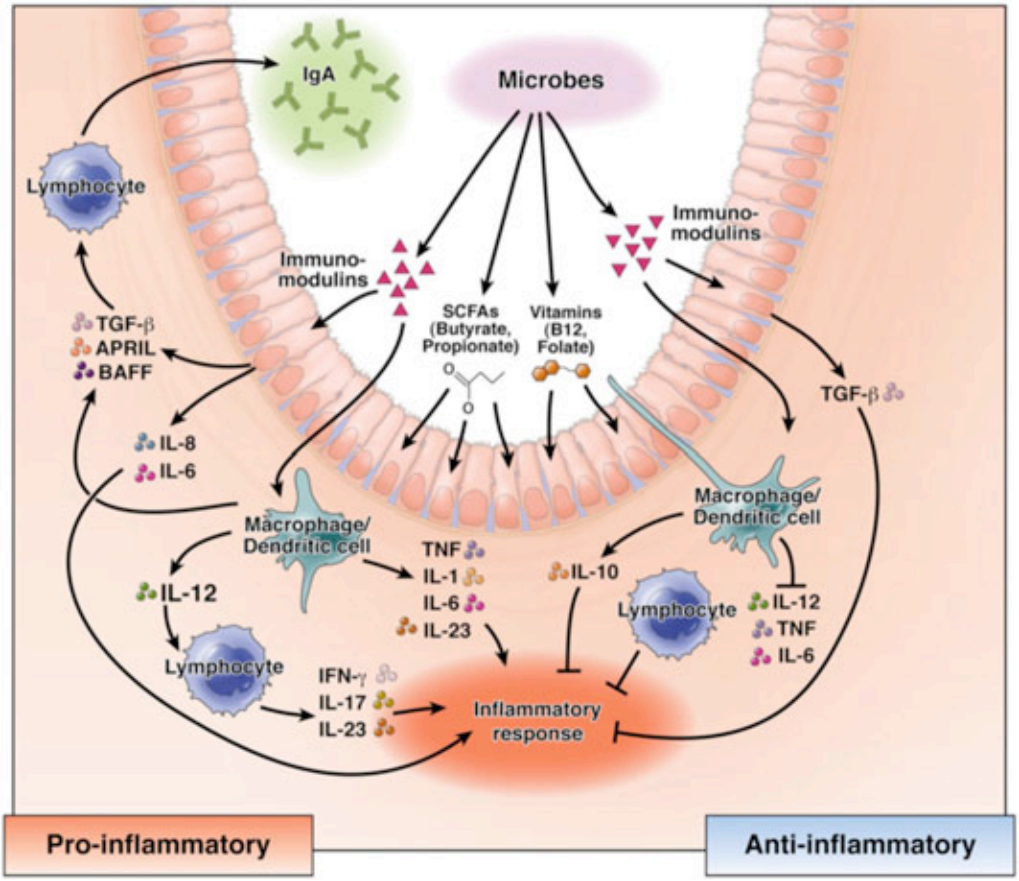

Figure 2: Immunomodulation by beneficial microbes. Probiotics can modulate intestinal immune system by production of secreted soluble factors and metabolites, such as short-chain fatty acids (SCFAs) and vitamins. These factors affect the function of the intestinal epithelium and mucosal immune cells, resulting in production of cytokine and related factors such as a proliferation-inducing ligand (APRIL) and B-cell activating factor (BAFF). Figure adapted from *Preidis* et al., 2009.

Beneficial microbes in the gastro-intestinal tract have been shown to modulate the intestinal immune system by production of secreted factors and metabolites that affect the growth and function of intestinal epithelial cells and immune cells (Figure 2) (*Preidis* and *Versalovic*, 2009).

L. reuteri regulates the intestinal immune system in several aspects and can be considered an "immunoprobiotic" (*Lin* et al., 2008). Recent *in vitro* and *in vivo* studies have demonstrated its role in host immunomodulation, along with the molecular mechanisms behind it. Interestingly, these activities seem to be highly strain-dependent (*Liu* et al., 2010; *Pena* et al., 2004), and can affect both innate and adaptive immune responses.

Several studies have demonstrated the ability of *L. reuteri* to regulate the activity of immune cells and the production of cytokines from these cells. Heat-killed *L. reuteri* 100-23 induced the production of anti-inflammatory cytokine IL-10 by bone marrow-derived dendritic cells (BMDCs)

(*Livingston* et al., 2009). When these *L. reuteri*-treated cells were incubated with splenic T-cells from ovalbumin T-cell receptor transgenic mice, IL-2 production was reduced and transforming growth factor-β (TGF-β) production increased. Moreover, spleens and mesenteric lymph nodes from *Lactobacillus*-free mice colonized with *L. reuteri* contained more FoxP3-positive cells than that of control mice. These results suggested that besides eliciting intestinal immune responses, *L. reuteri* also regulated the development and recruitment of regulatory T-cells to the gastrointestinal epithelium. *L. reuteri* may regulate intestinal inflammation by controlling recruitment of immune cells in a gnotobiotic neonatal pig model of rotavirus infection. In animals pre-colonized with human-derived *L. reuteri* ATCC 23272 and *L. acidophilus* NCFM, recruitment of monocytes and macrophages to the intestines and spleens was inhibited. This result suggested that colonization by these lactobacilli may reduce rotaviral infection-induced monocyte/macrophage recruitment to the intestine and systemic lymphoid tissue (*Zhang* et al., 2008).

Studies have also suggested that soluble factors from *L. reuteri* could inhibit production of proinflammatory cytokines and inflammatory signal processing in immune cells (*Thomas* and *Versalovic*, 2010). Cell-free culture supernatants from murine-derived *L. reuteri* 6798 were able to inhibit tumour necrosis factor (TNF) production by lipopolysaccharide (LPS)-activated (*Pena* et al., 2004) and *Helicobacter hepaticus*-treated (*Pena* et al., 2005) mouse macrophages. Moreover, culture supernatants from human-derived *L. reuteri* ATCC PTA 6475 demonstrated strain-specific suppression of human TNF production by activated monocytoid cells (THP-1) and primary monocyte-derived macrophages from patients with Crohn's disease. Transcriptional regulation of TNF expression by *L. reuteri* occurred by inhibition of c-Jun-dependent activator protein 1 (AP-1) pathway (*Lin* et al., 2008). Interestingly, *L. reuteri* formed biofilms, and biofilm cultures of *L. reuteri* PTA 6475 also suppressed TNF production by activated THP-1 cells (*Jones* and *Versalovic*, 2009). A recent comparative transcriptomic study identified a number of genes that might play a role in the production of such soluble factors (*Saulnier* et al., 2011a). Further characterization of these genes and their potential roles in immunomodulation is currently on-going.

The *in vivo* effects of *L. reuteri* in the human intestinal immune system was demonstrated in a small clinical investigation, which used *L. reuteri* ATCC 55730 to colonize the gastrointestinal tracts of healthy volunteers and patients with ileostomy (*Valeur* et al., 2004). After supplemented with *L. reuteri*, the numbers of duodenal B-lymphocytes and CD4-positive T-lymphocytes were significantly increased in the ileal epithelium. These observations suggested that *L. reuteri* may be able to regulate both humoral and cell-mediated aspects of the adaptive immune response in humans.

LACTOBACILLUS REUTERI AND THE INTESTINAL EPITHELIUM

Experiments using germfree animals and knockout mice lacking the Toll-like receptor (TLR) signal transduction pathway component MyD88 suggested that gut microbiota could influence the development and differentiation of the intestinal epithelium. Several studies have demonstrated that the intestines of

germfree mice were both morphologically and functionally underdeveloped in multiple aspects (*Smith* et al., 2007). Intestinal microbes also play an important role in maintaining the epithelial lining and integrity of the intestinal barrier. Germfree mice and MyD88-knockout mice had significantly decreased colonic epithelial cell proliferation after colonic mucosal injury compared to that of conventionally housed mice (*Pull* et al., 2005). Colonization of germfree mice with intestinal microbiota resulted in upregulation of genes involved in intestinal barrier fortification (*Hooper* et al., 2001). TLR2 signalling initiated by exposure of intestinal epithelial cells to peptidoglycan from bacteria also enhances the integrity of tight junctions, which are responsible for the maintenance of intestinal barrier integrity (*Chung* and *Kasper*, 2010).

Several *Lactobacillus* strains increased the integrity of the intestinal barrier, which could result in protection from loss of immune tolerance, gastrointestinal infections, irritable bowel syndrome and inflammatory bowel disease (*Lee* and *Bak*, 2011). *L. rhamnosus* GG was able to preserve tight junction architecture and expression of tight junction protein zona occludens-1 (ZO-1) in the presence of pro-inflammatory cytokines such as interferon-gamma (IFN-γ) (*Donato* et al., 2010). Treatment with *L. plantarum* CGMCC 1258 also resulted in amelioration of the loss of colonic paracellular integrity and restoration of expression and distribution of tight junction proteins (*Chen* et al., 2010). A recent study using a dextran sodium sulphate (DSS) colitis mouse model (*Zakostelska* et al., 2011) demonstrated that pre-treatment of animals with *L. casei* DN-114 001 resulted in protection against perturbation of intestinal permeability and barrier integrity, mainly by preserving ZO-1 expression in the mucosa of the terminal ileum and colon.

A recent study from our laboratory demonstrated that *L. reuteri* stimulated intestinal epithelial cell proliferation and differentiation in an outbred neonatal mouse model (*Preidis* et al., 2012a). Further studies are needed to reveal how *L. reuteri* affects the regulation of gene expression in the intestinal epithelium.

LACTOBACILLUS REUTERI AND PREVENTION/TREATMENT OF INTESTINAL INJURY

Several *Lactobacillus* species can facilitate prevention or treatment of intestinal injury caused by infection, excessive inflammation or radiation-induced reactive oxygen radicals. Acute radiation-induced intestinal injury commonly occurs in patients undergoing radiation therapy for malignancies, resulting in malabsorption, bloating, diarrhoea and dehydration (*Ciorba* and *Stenson*, 2009). Several preliminary studies have demonstrated preventative and therapeutic effects of probiotics on such injury in animal models. *L. delbrueckii* subspecies *bulgaricus* B3 was able to increase the villus/crypt ratio and the number of villi per square millimetre in the jejunum of gamma-irradiated mice, along with reduced inflammation and vascularity in all intestinal segments (*Demirer* et al., 2006). A recent study using a similar mouse model pre-treated with *L. rhamnosus* GG showed that probiotic treatment resulted in reduction of weight loss, intestinal cell apoptosis and crypt loss after gamma irradiation. Increased crypt survival was dependent on TLR-2, MyD88 and COX-2 signalling (*Ciorba* et al., 2011).

Crohn's disease (CD) and ulcerative colitis (UC) are two forms of inflammatory bowel disease (IBD) (*Rakoff-Nahoum* and *Bousvaros*, 2010). These debilitating diseases affect the quality of life of the patients worldwide, with highest prevalence rates in Israeli Jewish, North American and European populations (*Menon* et al., 2011). As mentioned in previous sections of the review, probiotics have been suggested to possess anti-inflammatory properties, strengthen the intestinal barrier and alter microbial-mucosal interactions. From these observations, it was hypothesized that probiotics may provide protection against intestinal inflammation (*Guandalini*, 2010). Experimental mouse acute colitis models have been used to study mechanisms of inflammation in the intestine and evaluate the efficacy of novel treatment modalities. Mice can lack the functions encoded by certain genes (such as IL-10, TGF-β, FoxP3) resulting in spontaneous colitis, or intestinal inflammation may be induced via chemicals such as dextran sodium sulphate (DSS), microbial infections (*H. hepaticus*) or hapten-producing compounds such as trinitrobenzene sulphonate (TNBS) or 4-chloro-7-nitro-2,1,3-benzoxadiazole (NBD-Cl) (*Ishiguro* et al., 2010; *Saleh* and *Elson*, 2011). Several probiotic strains have demonstrated beneficial effects in ameliorating intestinal inflammation in these animal models. In a DSS-induced mouse colitis model, treatment with *L. reuteri* BR11 was able to decrease disease activity index (DAI), distal colonic crypt hyperplasia and colitic symptoms compared to that of vehicle-treated mice (*Geier* et al., 2007). Moreover, pre-treatment of rats with a bacterial cocktail containing four strains of rat- and human-derived *L. reuteri* (R2LC, JCM 5869, ATCC PTA 4659 and ATCC 55730) resulted in reduction of mucosal damage and reduction of DAI. Downregulation of the adhesion molecule P-selectin was observed throughout the colon, resulting in decreased leukocyte-endothelial interactions in colonic venules of probiotic-treated animals (*Schreiber* et al., 2009). Interestingly, many other strains of lactobacilli, such as *L. casei* Shirota, *L. paracasei*, *L. plantarum* HY115 and *L. brevis* HY8401, yielded protective effects in similar DSS-induced colitis models as well (*Claes* et al., 2011). In a TNBS-induced rat colitis model, which is helpful in identifying the role of T-lymphocytes in colitis, animals were pre-treated with either *L. fermentum* CECT5716 or *L. reuteri* ATCC 55730 before induction of colitis. Both probiotics were able to demonstrate intestinal anti-inflammatory effects and reduced colonic TNF quantities (*Peran* et al., 2007). Similarly to the DSS-induced colitis model, several different *Lactobacillus* strains such as *L. fermentum* CECT5716, *L. acidophilus* IPL908 and *L. casei* BL23 have shown beneficial effects in alleviating the severity of disease in TNBS-treated animals (*Claes* et al., 2011; *Mane* et al., 2009). Moreover, recent unpublished data from our laboratory using fluorodeoxyglucose (^{18}F)-positron emission tomography (FDG-PET) suggested beneficial effects of *L. reuteri* ATCC PTA 6475 in a TNBS-induced mouse colitis model (Figure 3).

In terms of clinical evidence supporting the therapeutic use of probiotics in IBD, few randomized, placebo-controlled studies have been performed for treatment of CD, yielding largely no significant improvement in disease outcome (*Guandalini*, 2010). However, probiotics seem to perform better in treatment of UC. A recent study in paediatric patients suffering from active distal UC using *L. reuteri* ATCC 55730 administered as an enema showed a

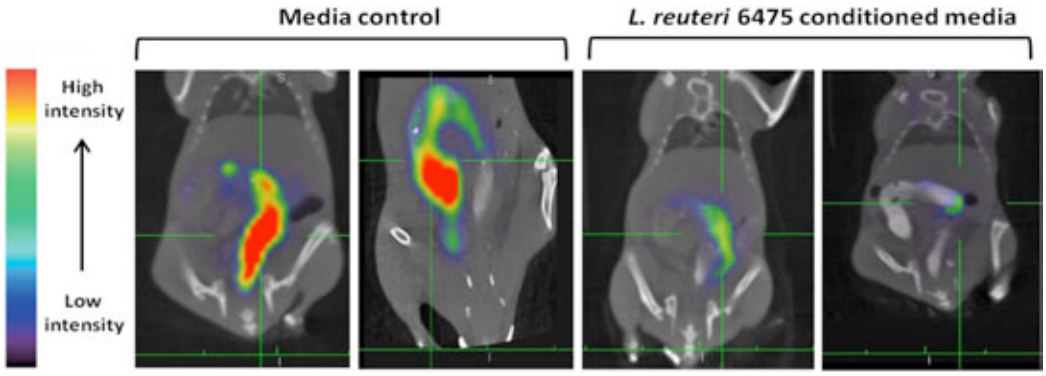

Figure 3: *In vivo* imaging suggested reduction of colitis in Balb/c mice treated with *L. reuteri* ATCC PTA 6475. Mice were treated with media control or conditioned media from *L. reuteri* 6475. Colitis was induced by trinitrobenzene sulfonate (TNBS). Fluorodeoxyglucose (^{18}F)-positron emission tomography (FDG-PET) revealed diminished signal intensity in colons of probiotic-treated mice compared to that of mice treated with a medium only control, suggesting that *L. reuteri* 6475 may have a protective effect against colitis in this animal model.

reduction in disease severity scores (clinically, endoscopically, and histologically observed) and reduced quantities of inflammatory cytokines in rectal tissue (*Oliva* et al., 2011). Since our previously mentioned study has identified *L. reuteri* ATCC PTA 6475 as an anti-inflammatory strain (*Lin* et al., 2008), it would be interesting to see how this strain would perform in a clinical study of similar design.

Bacterial and viral infections can also result in intestinal injury, which includes direct damage to the mucosa and epithelial lining, disruption of the intestinal barrier, and aberration of intestinal immune responses. Different probiotic strains of *Lactobacillus* yielded protective effects against severe intestinal injury in several animal models of gastrointestinal infections. In a study using a rat model of *Shigella dysenteriae* 1 infection, pre-treatment of animals with *L. rhamnosus* or *L. acidophilus* prior to infection resulted in amelioration of the loss of membrane-bound adenosine triphosphatase (ATPase) and tight junction proteins compared to that of vehicle-treated rats (*Moorthy* et al., 2009). The benefit of *Lactobacillus* in gastrointestinal infections was seen in an *Enterobacter sakazakii*-induced necrotizing enterocolitis (NEC) rat pup model. Animals pretreated with *L. bulgaricus* prior to induction of NEC demonstrated reduced nitric oxide production in the intestinal mucosa, a reduction of bacteraemia and improvement in survival compared to that of pups receiving no probiotics (*Hunter* et al., 2009). Beside the preventative effects, the potential therapeutic role of probiotics in recovery from infections was shown in a recent study using a rabbit model of *Staphylococcus aureus* enterocolitis. Young rabbits receiving fermented milk containing live *L. paracasei* after infection had decreased duration of diarrhoea, along with more rapid recovery of intestinal villi and colonic crypts (*Bendali* et al., 2011).

Rotavirus is the main aetiologic agent in acute gastroenteritis in children below one year of age worldwide, and causes more than 600,000 deaths worldwide per year (*Grandy* et al., 2010). A recent study from our laboratory demonstrated the effectiveness of *L. reuteri* DSM 17938 in the treatment

of rotaviral infection in a neonatal mouse gastroenteritis model (*Preidis* et al., 2012b). Results from animal models suggest that treatment with probiotics may serve as a low-cost and effective measure in prevention and treatment of gastrointestinal infections (*Preidis* et al., 2010).

INTESTINAL MICROBIOME AND HUMAN HEALTH: ROLE OF PROBIOTICS IN PREVENTION AND TREATMENT OF DISEASES

The human gastrointestinal tract is sterile *in utero*, but colonization of microbes begins at birth. Shortly after that, it becomes home to more than 10^{14} microbial cells, consisting of more than 1,000 different species which reside mostly in the colon (*Wallace* et al., 2011). The microbial population in each individual becomes relatively stable in terms of richness and diversity in early childhood after the weaning process (*Spor* et al., 2011). The majority of bacteria in the colons of adults are anaerobes, such as *Bacteroides spp.*, *Bifidobacterium spp*., *Clostridium* spp., *Eubacterium spp*. and *Lactobacillus spp.* (*Wallace* et al., 2011). As previously mentioned, the intestinal microbiota plays an important role in the function and integrity of the gastrointestinal tract, maintenance of homeostasis in the immune system, along with the energy metabolism of the host (*Pflughoeft* and *Versalovic*, 2011). Alterations in overall composition of microbial populations, also known as dysbiosis, can result in disruptions of the mutualistic relationships between microbe versus microbe or microbe versus host. These changes may affect the health of the host and result in potentiation of disease (*Frank* et al., 2011). Several reports have demonstrated associations between intestinal dysbiosis and energy harvest or metabolic disorders (*Jumpertz* et al., 2011), which can result in diseases such as metabolic syndrome, obesity and diabetes (*Claus* et al., 2008; *Larsen* et al., 2010; *Pflughoeft* and *Versalovic*, 2011). Alterations in the composition of intestinal microbiome have also been associated with gastrointestinal infections, inflammatory bowel disease, and irritable bowel syndrome (*Pflughoeft* and *Versalovic*, 2011; *Saulnier* et al., 2011b). Several current treatment modalities to manipulate and restore the balance in the richness and diversity of intestinal microbiome are currently being explored (*Sonnenburg* and *Fischbach*, 2011). One of the most studied approaches is the use of probiotics to introduce organisms with beneficial functions into gastrointestinal microbial communities, which may result in protection from or alleviation of diseases. Moreover, probiotics may be able to affect microbial communities by competition for nutritional substances or binding sites, production of growth substrates or inhibitors and modulation of intestinal immune response (*O'Toole* and *Cooney*, 2008). This concept is supported by results from randomized controlled clinical trials that studied beneficial effects of probiotics during the treatment of gastrointestinal diseases [extensively reviewed by *Preidis* and *Versalovic* (2009) and *Thomas* and *Greer* (2010)].

Scientists lack direct evidence regarding the impact of probiotics on the human intestinal microbiome in different human populations. With recent technological innovations in DNA sequencing and advancements in bioinformatics, we have entered the era of metagenomics. The Human Microbiome Project (*Peterson* et al., 2009) has

shaped the way scientists approach research questions related to the human microbiome and how each treatment modality can affect changes in the global composition of microbial communities. Probiotics induce changes in the intestinal microbiota and restore homeostasis in the gastrointestinal tract. However, further studies in humans are needed to explore whether probiotics can make the same impact on the human intestinal microbiome and whether such changes are associated with clinical benefits in the host.

CONCLUSION

A body of evidence has demonstrated beneficial effects of probiotic lactobacilli, including *L. reuteri*, on human health and disease. However, further studies must be done to fully understand the mechanisms of probiosis. Well-designed experiments should be performed in appropriate experimental models (*in vitro* or *in vivo*) and in the human host. Metagenomic, metatranscriptomic and metabolomic approaches should be used to globally examine interactions between probiotics and intestinal microbes and between probiotics and the mammalian host. Once exact mechanisms of probiosis have been identified in detail, efficient and safe probiotics may be engineered or selected as natural strains, resulting in novel preventive and therapeutic interventions for human intestinal disorders.

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BACTERIAL MICROBIOTA PROFILING IN STOMACHS WITH AND WITHOUT *HELICOBACTER PYLORI*

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SUMMARY

The human gut microbiota has come into focus in the search for component causes of chronic diseases, such as gastrointestinal cancer. Some scientists claim that *Helicobacter pylori* belongs to the normal bacterial flora from the human stomach, despite its associations with gastric ulcers and cancer in adulthood and that it seems to have beneficial effects in early ages. It has been shown that the stomach without *H. pylori* has a different bacterial composition beyond the absence of *H. pylori*. Factors that may shape the composition of the stomach microbiota are antibiotic treatment, diet, stomach acidity and histopathological changes i.e. development of atrophic gastritis in the corpus. Investigations of the bacterial microbiota in healthy human stomachs and changes in the microbiota due to factors mentioned above are necessary to rule out the impact of a well balanced microbiota in a healthy stomach and dysbiosis in gastric disease development. It has been suggested that other bacteria than *H. pylori* may be involved in pre-cancer lesions of the stomach. Next generation sequencing platforms give us a chance to explore the non-*H. pylori* microbiota in the stomach and will hopefully provide biomarkers for future studies of risk individuals.

INTRODUCTION

The human microbiota consists of about 100 trillion microbial cells that outnumber our human cells by 10 to 1 (*Savage*, 1977). Especially the human oral and gastrointestinal (GI) microbiota have been extensively studied but are yet not fully described. However, it has been established that the microbiota is partly site-specific (*Dethlefsen* et al., 2007). The human gastro-intestinal tract (GI-tract) consists of several compartments with varying physiological conditions and as a result different microbiota. On their passage through the GI tract the bacteria will be exposed to peristaltic activity, food particles, gastric-, pancreatic- and bile secretions at different locations of the tract. In the stomach and upper part of the small intestine the low pH, fast peristalsis and high bile concentrations will limit the bacterial colonization and survival (*Manson* et al., 2008). Further down in the colon the restrictive feature is the anaerobic environment and as a consequence the anaerobic bacteria outnum-

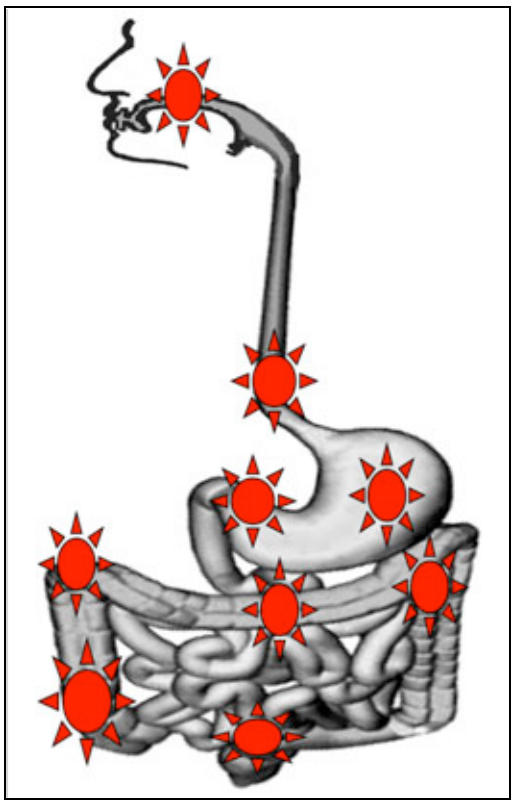

Figure 1: Sampling sites in the human gastrointestinal tract. Upper endoscopy allows sampling from the oesophagus, stomach and duodenum. Lower endoscopy covers the entire large bowel. The small intestine is not possible to sample using traditional endoscopy.

ber the aerobic by 1000:1. The different parts of the GI-tract that are reachable with the endoscope, thus allowing us to obtain biopsy samples from these locations, are shown in Figure 1. Today it is not possible to sample the entire small intestine and consequently the composition of the microbiota in this part of the human GI-tract is considered almost a black box.

Most of the microbiota in the GI-tract is located in the intestinal lumen or in the loose mucus close to the lumen without direct contact to the epithelium (*van der Waaij* et al., 2005; *Swidsinski* et al., 2007a). The mucus layer (which consists mainly of different mucins) in the stomach and colon is divided into the firmly attached mucus layer and the loose mucus layer. The mucus layer is a protective barrier that prevents epithelial colonization of microbes and prevents diffusion of damaging chemicals, including gastric acid, to the epithelial surface. Thus, inflammation in the GI-tract is caused either by bacterial penetration of the mucus layer or by a disruption of the same layer.

With new molecular-based methods the information about the microbiota in the GI tract is constantly growing and provides deeper understanding on microbial ecology and the involvement of the microbiota in health and disease. Most studies are based on 16S rRNA gene sequencing methods and the results are descriptive but not functional.

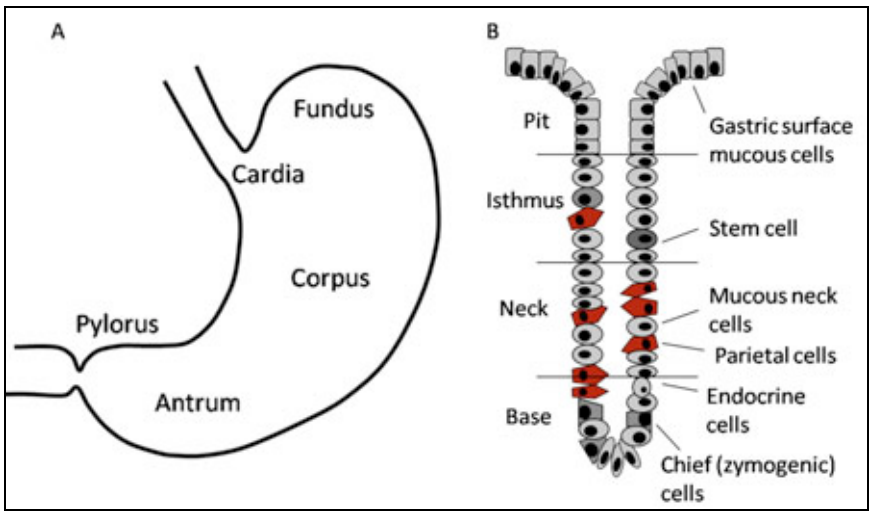

Figure 2. A. Illustration of the human stomach anatomy. B. Schematic view of the gastric unit.

In the near future when sequencing costs will go down, GI tract microbial metagenome studies will probably replace or complement the 16S-based studies. We will then get information about sets of core genes (including functional genes) specific for the gut microbiota and associations of these genes with disease development. The putative gut-specific genes include genes involved in adhesion of host proteins and harvesting of sugars (*Qin* et al., 2010).

REVIEW AND DISCUSSION

The oral and oesophageal microbiota

Despite the presence of antimicrobial peptides and the constant flow of saliva, the oral cavity has a very high abundance of microorganisms with 10^9 bacteria per ml saliva and 10^{11} bacteria per gram dental plaque (*Aas* et al., 2005). The mouth has both soft and hard tissue and different species seem to preferentially colonize either of the tissue types. Great similarities have been found in the oral microbiota in different individuals and these species and genera have been identified as the core microbiome. The most prevalent genera and families in this oral core microbiome are *Streptococcus*, *Corynebacterium*, *Neisseria*, *Rothia*, *Veillonellaceae*, *Heamophilus*, *Actinomyces*, *Granulicatella* and *Prevotella* (*Zaura* et al., 2009). It would be expected that these genera follow the swallowed saliva down into the stomach. In biopsies obtained from oesophagus, a similar but more sparse biota has been found, with *Streptococcus*, *Rothia*, *Veillonellaceae*, *Granulicatella* and *Prevotella* as the most prevalent genera (*Pei* et al., 2004).

The gastric microbiota

Anatomy and physiology

A meal can take only minutes to eat, but takes hours to digest, therefore, the main function of the stomach is to store food and send gastric contents ahead with a speed that maximizes digestion in the small intestine. Some di-

gestion of proteins and starch takes place in the stomach. Under fasting conditions the pH in the human gastric lumen is < 2 and is about 5-6 close to the surface epithelial cells. The pH gradient is monitored by the mucus layer that covers the epithelial cells in the stomach. MUC1 is a transmembrane mucin and constitutes the main factor in the firmly attached mucus layer. The other mucins are secreted mainly to the loose mucus and the most secreted is MUC5AC (*Corfield* et al., 2001).

An adult person produces 2 litres of gastric juice daily. Most of the juice is formed in the tubular glands in the gastric corpus- and fundus-regions. The glands form deep pockets into the gastric wall and comprises of different cell types (Figure 2).

The gastric microbiota in health

Even though the human microbiota along the intestinal tract has been extensively studied, the environment in the stomach has been considered too harsh for most bacteria to thrive and survive in for any length of time and therefore this environment has not been studied to any great extent. However, since the discovery in 1984 of *H. pylori* as the causative agent of peptic ulcers and a risk factor for the development of gastric cancer, this specific bacterium has been extensively studied.

In the normal acidic stomach a sparse cultivable non-*Helicobacter* microbiota has been found dominated by *Veillonella* sp., *Lactobacillus* sp., and *Clostridium* sp. (*Zilberstein* et al., 2007). A more diverse microbiota has been seen when using 16S rRNA based methods and the main genera found in stomachs except *Helicobacter* have been, *Streptococcus*, *Prevotella*, *Veillonella* and *Rothia* (Figure 3, *Li* et al., 2009). Whether these other genera belong to a colonizing microbiota or are just swallowed bacteria from the oral cavity has not yet been determined. The main stomach microbiota consists of the same genus as found in the oral cavity and also in oesophagus biopsies (*Keijser* et al., 2008). However, it is not a direct reflection of these microbiota. The gastric microbiota is well adapted to the gastric environment and also to environmental changes in their specific stomach.

The gastric microbiota in disease

Helicobacter pylori is a micro-aerophilic, spiral shaped Gram-negative rod and is today the only bacterium considered to colonize the human stomach. *H. pylori* survives in the acidic gastric lumen by production of urease that coverts urea to ammonia and carbon dioxide, which neutralizes the microenvironment close to the bacterium. As a result of the urease activity the pH around the bacteria will increase, leading to reduced viscosity and enables *H. pylori* movement trough the mucus (*Celli* et al., 2009). *H. pylori* can adhere to the gastric epithelial cells but the majority (99%) are free-living in the mucus (*Falk* et al., 2000) and especially in association with mucin MUC5AC, which is the most common mucin in the gastric mucus layer (*Van de Bovenkamp* et al., 2003). When *H. pylori* is present in the stomach it totally dominates the gastric microbial population in the gastric niche constituting as much as 94% of the total number of sequences in one individual (Figure 3). When the number of *H. pylori* diminishes, for example during atrophy development in the gastric niche, the diversity increases dramatically.

Helicobacter pylori induced atrophic gastritis and gastric cancer

Helicobacter pylori colonizes almost 40% of the population in many western countries. Most individuals

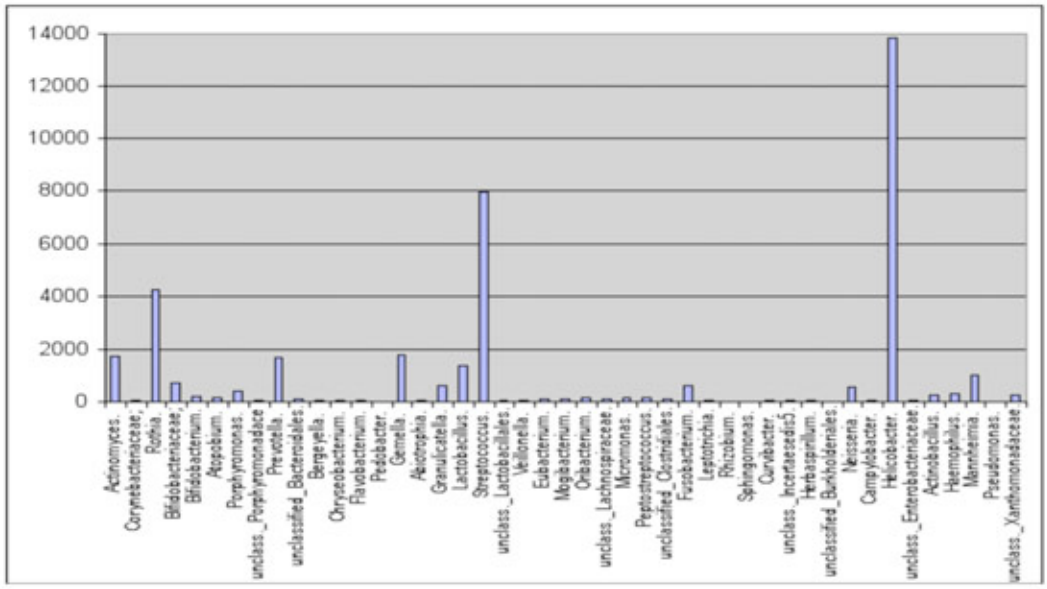

Figure 3: The most prevalent bacteria in the human stomach (number of reads sampled).

with gastritis will have an asymptomatic infection but about 10-20% of the infected individuals will develop ulcers and 1-2% will develop cancer (*Kusters* et al., 2006). The outcome is depending on the location of infection. Patients with antral-predominant gastritis are predisposed to duodenal ulcer, whereas patients with corpus-predominant gastritis are more likely to develop gastric ulcer, atrophic gastritis, intestinal metaplasia and gastric cancer. The low acid production in corpus-predominant gastritis is due to atrophy development since chronic *H. pylori* induced inflammation during decades can lead to such changes in the gastric mucosa. In corpus-dominated atrophy the acid-producing parietal cells have been changed into more intestinal like non-acid producing cells resulting in intestinal metaplasia and a less acidic stomach. The change in cell types also changes the mucin production in the stomach to the intestinal mucins MUC2 and MUC3 (*Babu* et al., 2006). Because bacteria often bind to different mucins, this change can affect how well and which bacteria adhere to the mucus. *H. pylori* has been found to spontaneously disappear in patients with severe corpus-predominant atrophy where the bacteria gradually disappear as the atrophy worsens. The disappearance of *H. pylori* and low acid output due to atrophy opens up for other non-*H. pylori* bacterial populations to invade into this niche (Figure 4).

The gastric microbiota in corpus predominant atrophic gastritis

The atrophic stomach has an increased pH enabling better survival of bacteria than in a normal acidic stomach. In addition a shift can be seen in the most prevalent genera from *Prevotella* to *Streptococcus* in the atrophic stomach. This shift further confirms that the gastric microbiota is not only a reflection of the oral microbiota. The bacteria with the greatest increase in the atrophy group represent the mitis group within the *Streptococcus* spp. In a study where *H. pylori* negative individuals with antral gastri-

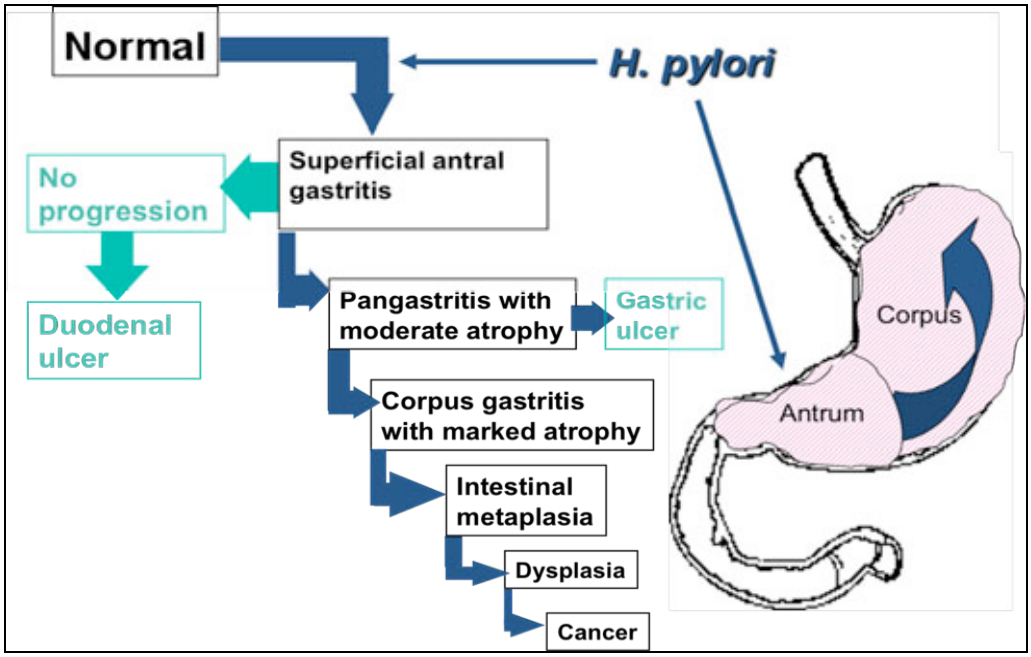

Figure 4: Gastric cancer development and *H. pylori* distribution within the stomach. Curved arrow indicates expansion of the *H. pylori* population into the corpus area when pH increases. Increased pH also allows for non-*H. pylori* bacterial populations to survive in the non-acidic stomach.

tis (where the pH should be expected to be decreased if changed) was compared to non-symptomatic controls (*Li* et al., 2009). In this study shifts could be observed in the antrum biopsies (in *Prevotella*, *Streptococcus* and *Rothia*) as described for the corpus predominant atrophy stomach.

The gastric microbiota in gastric cancer

As in the atrophic stomach, the pH is increased in gastric cancer and both conditions can also lead to an increased amount of bacteria and increased diversity. In gastric cancer the number of bifidobacteria/lactobacilli, *Veillonella* and streptococci are increased (*Sjöstedt* et al., 1985). Among the *Streptococcus* especially a group including *S. mitis* and *S. parasanguinis* increases. The changes in the microbiota in individuals with gastric cancer resemble those seen in the atrophic stomach.

The gastric core microbiome

The gastric microbiota is totally dominated by five phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria*) and each phylum comprises of only a few genera all in high abundance. In the same way as a core microbiome for the oral cavity has been determined (*Zaura* et al., 2009) it is possible to determine a core microbiome for the healthy human stomach. We determined that, among 13 individuals with no pathology and without dominance of *Helicobacter* sequences, the majority of the bacteria were represented in all individuals (unpublished data). The most prevalent bacterial sequences belonged to *Streptococcus*, *Prevotella* and *Veillonella*. In addition the five most abundant genera from two other studies (*Bik* et al., 2006; *Li* et al., 2009) were all among the ten most abundant in our study. These

similarities can be seen regardless that different approaches for DNA extraction, primer design and sequencing were used in the different studies. Taken together this is an indication that the core gastric microbiome is dominated by ten genera. In addition, it has been found that there are no big differences in the microbiota present in the antrum and corpus portion of the stomach (*Bik* et al., 2006; *Li* et al., 2009) with the exception of the higher proportion of *Prevotella* in antrum found by Li and colleagues (*Li* et al., 2009). On the whole, the most prevalent genera in the healthy gastric core microbiota are *Prevotella*, *Streptococcus*, *Veillonella*, *Rothia*, *Haemophilus*, *Actinomyces*, *Fusobacterium*, *Neisseria*, *Porphyromonas* and *Gemella*.

In conclusion, the human gastric microbiota has been investigated and great inter-individual similarities are found in healthy stomachs, with the microbiota dominated by five phyla and ten genera. In the unhealthy stomach a significant shift is observed in stomachs with corpus atrophy compared to controls, with *Streptococcus* becoming the most abundant genus that may affect disease development. It is however important to understand the relative contributions of the host and environmental factors to the dynamics of the stomach microbiota. High-throughput sequencing platforms will allow us to study the gastric metagenome in the near future and such studies will provide information about the functional repertoire of genes and the expression of these genes. We will also increase our knowledge of metabolic products of active microbial populations (in a healthy compared to a diseased state). Future research in this field will require well-designed prospective epidemiological studies combined with optimized sampling procedures and access to front-line technology platforms.

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Mathilda Lindberg is acknowledged for her thesis work on this subject.

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HELICOBACTER – A VERSATILE PATHOGEN AND A VANISHING SPECIES IN THE STOMACH

(The evolution of stomach microbiology in the antibiotic era)

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SUMMARY

Helicobacter pylori is a highly versatile pathogen with a coevolution of the stomach in mammals, birds and other animals. *H. pylori* is closely related to enterohepatic, bile-tolerant species (EHS), colonizing the gut of most wild rodents, felines and several other species. This evolution is complex, and the primates seem to be the environment for *H. pylori* to develop before the exodus of man from Africa about 55 000 years ago. The cagA pathogenicity island (PAI) encoding for a series of surface proteins and virulence factors (cagA to E) is unique for *H. pylori* and strongly associated to its success as a pathogen, which in turn is strongly related to a number of human diseases, i.e. type B gastritis, duodenal and peptic ulcer disease (PUD), and gastric carcinoma. Aggressive treatment strategies and screening programmes have resulted in a decline of *H. pylori* in the Western world with a concomitant decline in PUD and gastric carcinoma. The unique human-to-human transmission explains the rapid associated decline in *H. pylori*-infections in children in the Western world but not in non-industrialized societies. There, gastric carcinoma is still the second most common cancer.

Possible "non-antibiotic" strategies, such as probiotic therapy to treat *H. pylori*, and "the empty stomach" after eradication of *H. pylori* will be discussed.

INTRODUCTION

In the whole Western world and some rapidly developing Asian societies we notice a rapid change of diseases with a decline of antibiotic-curable infections and a simultaneous increase in a number of allergic diseases including allergic childhood asthma, rhinitis, eczema and other skin disorders, often proposed to be related to a "too clean" lifestyle (Figure 1) (*Chen* and *Blaser*, 2008). An infection and antigen-rich environment may be essential for maturation of a normal immune system, preventing allergies, asthma and autoimmune diseases related to disturbances of a skin- and mucosa-associated microbiota in genetically prone individuals. An infection in the gastrointestinal tract (GIT) with the gut-associated lymphoid tissues, GALT, is related to

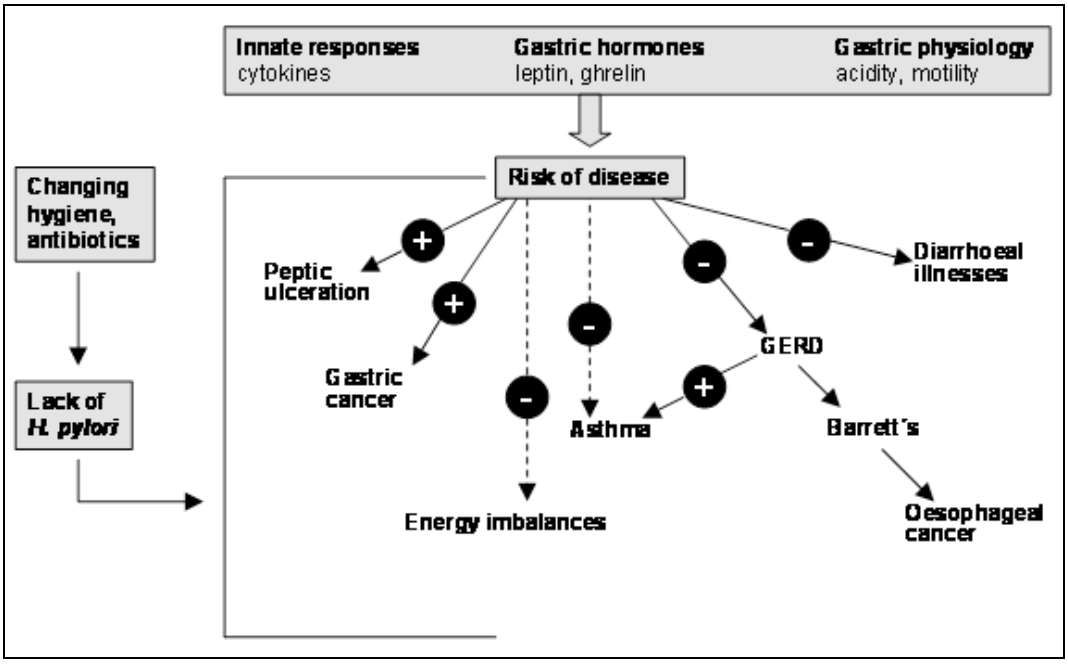

Figure 1: Who are we? Indigenous microbes and the ecology of human diseases.
[Adapted from: Blaser, M.J.: EMBO Rep. 7, 956-960 (2006)].

antibiotic use and overuse worldwide (*Vakil* and *Mégraud*, 2007).

Helicobacter pylori colonizes the human stomach shortly after weaning until recently, when modern living habits and common antibiotic use in early childhood prevent this colonization process. This fact was probably favoured by an early rotavirus and other enteric and respiratory infections. A transient pH rise in these infections may favour the gastric colonization to avoid the normal acid barrier. The impact of other factors such as diets after weaning as well as malnutrition and smoking with an alkaline stomach pH is still poorly understood (*Stenström* et al., 2007).

However, *H. pylori* gastritis studies in mouse and Mongolian gerbil models have been most useful to expand our understanding of the development of an acute, sub-chronic into chronic infection. *H. pylori* infections are always localized to the gastric mucosa in the stomach as well as to gastric tissues in other parts of the GIT, such as the Meckel's diverticle (*Haesebrouck* et al., 2009).

We know from extensive sero-epidemiological studies that *H. pylori* appeared in the human stomach in our ancestors in Africa > 58,000 years ago (*Linz* et al., 2007), is present in all human populations worldwide today, and acquired early after weaning. *Casswall* and co-workers (1999) analysed stool samples in young children in Bangladesh and showed that a transient colonization was followed by a later infection with a seroconversion in ELISA for urease and cell surface proteins (CSP). Early infections in some paediatric populations may affect appetite and food nutrient uptake, and induce a shunting syndrome in a subgroup, probably first reported by Oderda and colleagues in a South

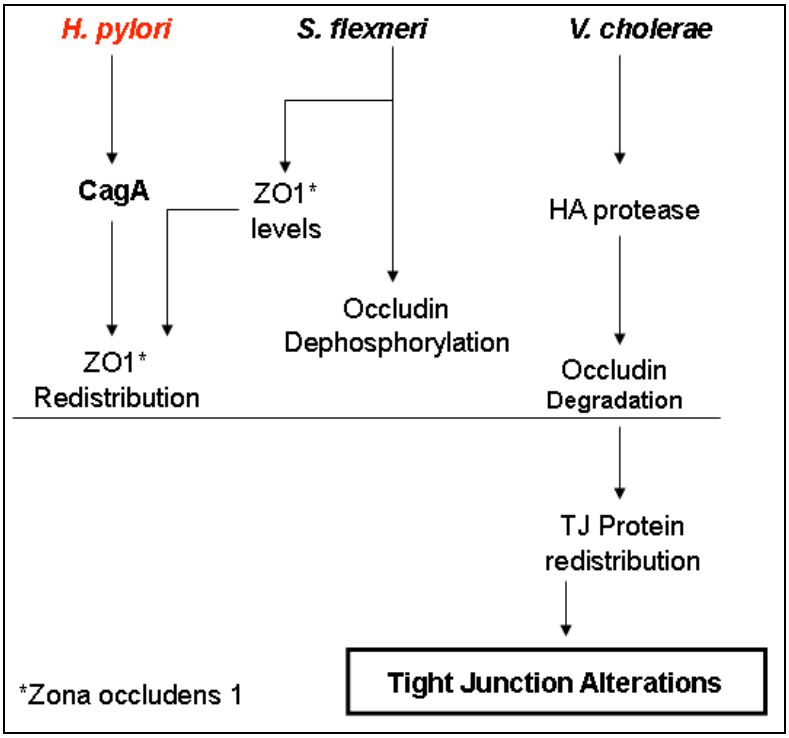

Figure 2: Disruption of tight junctions by microbes and microbial products.

Italian study (*Mégraud* and *Malfertheiner*, 2010). The majority of these infections are symptom-free (i.e. silent) but induce a local and **severe** immune response unlike a number of transient gastric colonizing microbes from food or the oral cavity. In brief, *H. pylori* is the single and dominant member of the human stomach microbiota (*Del Prete* et al., 2008). It penetrates the mucus layer, colonizes the epithelium, and pass cellular tight junctions into the sub-epithelial extracellular matrix (ECM), and binds to specific components by cell surface proteins (CSP:s) and LPS such as laminin and collagen type IV (Figure 2) (*McGuckin* et al., 2010).

HELICOBACTER AND MAN: A CO-EVOLUTION STORY

Mucus layer colonization and immune evasion

Gastric and enterohepatic Helicobacter species (EHS) colonize the mucus layer like other micro-aerophilic microorganisms such as *C. jejuni*. However, *McGuckin* and co-authors (2011) emphasize that microbes such as the indigenous microflora but not pathogens, rarely penetrate to the mucin deep layer. However, a chronic colonization and immune evasion is most characteristic for *H. pylori* unlike many animal Helicobacter species, such as *H. felis* (Table 1), rarely causing a chronic infection similar to *H. pylori* gastritis. *H. pylori* has developed a strategy to minimize stimulation of Toll-like receptors (TLRs), such as TLR-5 recognizing flagellae of *Salmo-*

Table 1. Enteric *Helicobacter* species isolated or detected in humans

Bacterial cultivation and/or PCR detection species	Cause/association in man	Animal host
H. canadensis	Gastroenteritis	Goose
H. winghamensis	Gastroenteritis	?
H. pullorum	Gastroenteritis, bacteraemia	Poultry
H. cinaedi	Diarrhoea, bacteraemia	Macaque, dog, hamster
H. sp flexispira	Bacteraemia	Pig, sheep, dog, cat
H. fennelliae	Bacteraemia, septic shock	?
H. bilis	Cholecystitis	Mouse, rat, dog, cat
H. hepaticus	Chronic liver disease	Mouse, hamster
H. rappini	Cholecystitis	Sheep
H. canis	Chronic liver disease	Dog, cat?

nellae but not of *H. pylori* (*Blaser* and *Atherton*, 2004). TLR-9 recognizes unmethylated DNA of most bacteria but not the highly methylated *H. pylori* DNA which minimizes recognition. *H. pylori* LPS is anergic, compared to enteric pathogens, related to lipid modifications and with poor reaction in classical LPS bioassays, such as the limulus test (*Moran* et al., 1992; *Hynes* and *Wadström*, 2004). *H. pylori* LPS is camouflaged from innate immune responses on cell surfaces. However, cagA positive strains stimulate NF-κB activation in the epithelium and phagocytes with subsequent induction of pro-inflammatory cytokine production (*Yanagisawa* et al., 2005). The relative scarcity of *H. pylori*-associated disease in non-industrialized countries despite a high *H. pylori* prevalence may be due to predominant Th_2 responses among black Africans, often chronically infected by intestinal parasites, helminths and malaria (the African enigma) (*Linz* et al., 2007; *Del Prete* et al., 2005)

Effects of *H. pylori* on leptin and ghrelin.

Gastric leptin levels are higher in *H. pylori*-infected adults than in uninfected individuals (*Konturek* et al., 2006). In an animal model, *H. pylori* infection is associated with up-regulation of ghrelin and several adipocyte genes (i.e. adipsin, resistin, adiponectin etc.), and likewise in a study from Poland (*Plonka* et al., 2006). Weight gain is common after *H. pylori* eradication, maybe related to the obesity epidemic in many Western societies and to a rapid decline in *H. pylori* infections (*Chen* and *Blaser*, 2008).

Chronic gastritis and carcinogenesis.

Carcinomas arise in stomachs with pangastritis (intestinal type, metaplasia, dysplasia). *H. pylori*-infected neutrophils release oxygen free radicals (ROS), and ascorbic acid levels are low in *H. pylori*-infected stomachs. Gastric cancer (GC) is number two of all cancers worldwide, increasing in African countries with an ageing population but declining in Western Europe related to an efficient eradication program (*Vakil* and *Mégraud*, 2007). The importance of dietary carcinogens in these malignancies is not well understood. However, food and snuff carcinogens act in synergy in *H. pylori*-infected mouse stomachs and tumours develop

regularly (*Stenström* et al., 2007). Well-developed mouse models are now most relevant for further studies of microbes, food and genetic background interactions in gastric cancer, similar to the more complex colon microflora-food interaction and colon cancer.

Probably all mammalian species including whales and dolphins seem to be colonized in the stomach by Helicobacter or Helicobacter-like organisms (*Haesebrouck* et al., 2009). However, several species studied in the early "Helicobacter history", such as *H. felis* in cats and dogs, migrate freely in the mucus layer but cause no or transient mucosal penetration in contrast to *H. pylori* in man (*Marshall,* 2002). However, *H. mustelae* in ferrets and *H. suis* in piglets colonize the gastric mucosa and cause mucosal ulcers much like *H. pylori* with the cagA pathogenicity island (PAI) and vacuolating toxin. *Candidatus Helicobacter suis* is a spiral bacterium in the stomach of pigs. Experimental infections in pigs have fulfilled the Koch's postulate with lymphoid follicle proliferation and a mild inflammation mainly in the antrum close to the parietal cells, as described for *H. pylori* in humans (*Haesebrouck* et al., 2009). However, the mild infection is making this model less likely to be developed for further studies of Helicobacter/gastrospirillum pathogenesis. Some epidemiological studies in China indicate that an early *H. suis* infection may protect against a *H. pylori* infection by unknown mechanisms of interference with a lower GC incidence in the population (*Haesebrouck* et al., 2009).

Several outer membrane proteins (OMPs) have been identified, like mucus-binding molecules including a neutrophophil binding and activating protein, or HPNAP (*Parker* et al., 2010). In this *H. pylori* OMP gene family (*Oleastro* et al., 2010) single or two copies of the homA/homA, homA/homB, or homB/homA and homB were found to be associated with gastro-duodenal disease with gastric inflammation and stomach atrophy, and identified as tissue adherence factors and implicated in IL-8 secretion. On the opposite, the homA gene is not associated with gastric inflammation.

H. pylori displays the highest level of genomic variability in bacteria with a high frequency of recombination contributing to the great diversity (*Linz* et al., 2007). Intragenomic recombination in the OMP genes occurs during a chronic *H. pylori* infection, probably reflecting a selective pressure for adhesins to adapt to the individual host and variations in the immune responses. *H. pylori* polymorphism reflects human phylogeography and human migrations, a most reliable marker of host pathogen co-evolution, facilitated by the long-term contact between the infecting strain and the host. Resulting genetic combinations seem to be critical for the ecological success of *H. pylori* strains (*Oleastro* et al., 2010). This great complexity in antigenic variations is a great challenge for *H. pylori* vaccine research.

H. PYLORI AND ANTIBIOTIC RESISTANCE

Suboptimal antibiotic eradication of *H. pylori* with up to a new quadruple therapy to treat patients to eradicate *H. pylori* upon several therapy failures confirmed by urea breath testing (UBT), gastroscopy or serology is a new challenge to develop alternative therapies (*Vakil* and *Mégraud*, 2007).

Cazzato and co-authors (2004) proposed to combine an antibiotic regime with a probiotic, i.e. Lactic Acid Bacteria (LAB) treatment to prevent side ef-

fects such as gastric reflux disease. *Sakamoto* and co-authors (2001) characterized a probiotic strain of *Lactobacillus gasseri,* and developed a Japanese yoghurt at the Meiji company to suppress *H. pylori* stomach infection as shown by UBT. Michetti and co-workers (1999) showed by UBT that another probiotic strain of LAB, *L. acidophilus* La1, inhibits the infection. Interestingly, LAB in the murine fore stomach inhibits the *H. pylori* infection in C57 Black and BALB/c mice (*Wang* et al., 1997), while germfree mice are more easily infected by a cagA positive strain of *H. pylori* devoid of LAB microflora in the upper stomach.

LAB and Bifidobacteria produce a number of antimicrobial activities (AMA), including peptides, which may inhibit *H. pylori* as well as a number of other pathogens such as *Clostridium difficile* (*Ljungh* and *Wadström*, 2009). This may be important in the future with a rapid increase in antibiotic-associated diarrhoea (AAD), sometimes as a severe complication upon a *H. pylori* antibiotic eradication therapy including clarithromycin or a quinolone (*Szajewska* et al., 2009). Thus, probiotics may be a useful adjunct with increased rates of eradication with an increase of 77 % to 80 % in one study (*O'Connor* et al., 2010).

THE STOMACH – AN EMPTY MICROECOLOGY NICHE AFTER *H. PYLORI* ERADICATION

An increase of gastro-duodenal reflux disease, or GERD, occurs in all Western societies today, associated with a rapid decline *H. pylori* infections in children varying from 33% to 7% in some epidemiological studies (Figure 1) (*Chen* and *Blaser*, 2008). One study demonstrated that *H. pylori* is 600 times more abundant in vomit samples compared with stool samples, and that vomiting is an important factor for transmission among children, e.g. in day care centres.

THE EVOLUTION OF *H. PYLORI*, OTHER GASTRIC AND ENTERIC HELICOBACTER SPECIES

A few years before Marshall and Warren published the Nobel Prize winning original reports in 1983 on *H. pylori* as the cause of type B ("bacterial") gastritis (Table 2) while *H. felis* and other related spiral-shaped organisms colonize rodents, cats and dogs (Table 1) (*Haesebrouck* et al., 2009). Studies in monkeys and other animals have confirmed that *H. pylori* is a unique human pathogen. The closest species to *H. pylori* may be a gastric species reported in beluga whales and dolphins (*Haesebrouck* et al., 2009). Genetic analyses of *H. pylori* in various populations confirmed a close relation to the human exodus from Africa 58.000 years ago (*Linz* et al., 2007). The great number of newly discovered species and new species "in the pipeline" of enteric Helicobacter in wild and laboratory mouse colonies suggest a complex evolution from unknown ancestor(s) closely related to *Wollinella* (Table 1).

Eradication of *H. pylori* from the human stomach may be in close analogy to another strictly human pathogen, i.e. the smallpox virus. However, the drastic fall in colonization rates in

Table 2: The discovery of *Helicobacter pylori*

Year	Author(s)	Discovery
1893	Bizzozero	Spirochetes in dog stomach
1906	Krienitz	Spirochetes in the stomach with gastric cancer
1917	Dragstedt	Bacteria do not induce gastric ulcer
1938	Doenges	Spirochetes induce gastritis in monkeys and humans
1979	Warren	Spiral bacteria in the human stomach
1983	Marshall	*H. pylori* isolated and cultured
1985-1987	Marshall/Morris	Oral feeding with *H. pylori* proved Koch's 3rd postulate by Marshall and Morris

young children in US, EU and other highly developed human societies today will never reach zero, as for smallpox. Moreover, we never experienced an "empty" sterile human mucosal surface. We know from studies in obesity patients before and after gastric bypass operations that oral microbes as alpha streptococci and enteric organisms (as various enterococcal species) can cause overgrowth in the stomach in chronic type A and B gastritis. A few new *Lactobacillus* species, like *L. kalixanda* (*Roos* et al., 2005) in gastroscoped human volunteers, and a few acid-tolerant *L. salivarium* and *L. reuteri* were reported recently.

It seems likely that we can develop a new probiotic concept to encourage use of stomach-adaptable non-virulent microorganisms to fill this empty niche. Since the borderline plastic pathogen and *H. pylori* probably now continues to decline and disappear it seems likely that a new concept discussed by B Marshall to develop a non-virulent attenuated *H. pylori* strain as a vaccine delivery system may also become a future probiotic candidate in analogy with *E. coli* strain Nisslé in ulcerative colitis (*Ljungh* and *Wadström*, 2009).

Finally, studies on the stomach microbiota should continue to explore the pathogenic properties in pigs (*H. suis*) and the complex gastric microflora in dogs and cats. The fore-stomach of these animals as well as of horses and rodents are colonized with LAB (*Haesebrouck* et al., 2009). These should be eradicated to permit a successful Helicobacter experimental infection (*Wang* et al., 1997).

HELICOBACTER AND CANCER

Marshall and Warren already in 1983 proposed that *H. pylori* was causing chronic type B (bacterial) gastritis and was an important vector in the development to atrophy and gastric metaplasia to dysplasia and gastric cancer, or GC (*Marshall*, 2002). The development of combined proton pump inhibitors´ (PPI) and antibiotic therapies now rapidly changed the GC epidemiology in societies all over the world. With a rapid decline in *H. pylori* infections in early childhood the person-to-person spread is rapidly declining. Without a chronic *H. pylori* gastritis at least 80-90 % of GC will be prevented, previously calculated to occur in about 11 % of *H. pylori* type B gastritis, thus a stronger

cancer risk factor than smoking in lung cancer (*Stenström* et al., 2007). Since most *H. pylori* infections are silent, a strategy to screen for GC with serology and gastroscopy with histopathology and culture, and PCR diagnosis of *H. pylori* infections are discussed, most recently at the European *H. pylori* conference in Florence, November 2010 (see: www.helicobacter.org).

In brief, it seems most appropriate to develop a pan-European screening program to diagnose *H. pylori* infections in our aging population with special attention to high *H. pylori* infection rates in South and East Europe and in immigrants from Turkey and other countries in the Middle East and in Africa. GC is still number four of all cancers worldwide and increasing in Indonesia and some other Asian countries with a rapidly growing young population, living in poor sanitation conditions. Interestingly, Canada decided already a decade ago to perform *H. pylori* serology on all immigrants applying for a Canadian citizenship (*R. Hunt*, McMaster University, personal communication). Thus, a *H. pylori* vaccine and other prevention methods seem to be a most attractive strategy to fight peptic ulcer disease and stomach cancer in Asia and Africa. B Marshall's laboratory in Perth is developing a new vaccine concept based on an attenuated *H. pylori* strain as a candidate to carry other vaccine candidates for an oral to stomach vaccine delivery.

HELICOBACTER, ANIMAL MODELS AND CANCER

The development of models to study *H. pylori* infection in mice and Mongolian gerbils are important tools to evaluate vaccine candidates and other strategies to prevent and treat *H. pylori* and other Helicobacter infections (see below). A number of dietary components such as antioxidants suppress these infections, and a low antioxidant diet enhances them. Recently, also some EHS, such as *H. bilis*, *H. pullorum* and *H. hepaticus* were shown to cause a chronic gut, biliary tree and liver infection in specific mouse models as the IL-10-/- mouse. We isolated *H. ganmani* in a spontaneous outbreak of IBD-like disease in IL-10 -/- mice with a histopathology similar to ulcerative colitis and sclerosing cholangitis in humans (*Nilsson* et al., 2008). These findings are also supported as new disease syndromes in a mouse model to study bile stone chronic cholecystitis in hyperlipemic mice (Maurer *et al.*, 2005). Likewise, they support early observations of chronic cholecystitis related to a *Salmonella* and *C. jejuni* biliary tract infection, probably very common in many non-Western societies**.** A possible link between Helicobacter infections and chronic cholangitis and bile stone disease is under investigation in several laboratories (*Nilsson* et al., 2005; *Pandey* et al., 2009; *Karagin* et al., 2010).

More research on a relation between EHS, biliary disease and IBD is certainly needed!

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SEGMENTED FILAMENTOUS BACTERIA AND INCREASED RESISTANCE TO INFECTIONS

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SUMMARY

Association of germfree mice with a complete intestinal microbiota promotes the development of the gut mucosal immune system, but this effect is not seen with individually cultured bacterial strains. *Clostridium*-related, segmented filamentous bacteria (SFB) are non-cultivable, commensal bacteria that strongly adhere to the epithelial cells of the ileum and of Peyer's patches. Their presence in mice stimulates formation of goblet cells, promotes intestinal transit, and results in MHC class II expression on the epithelium. Further, SFB stimulate IgA-producing cells in the lamina propria of the small intestine and activate T-cells, including T helper 17 (Th17) cells. As a consequence of this immunoactivation, adherence of SFB itself declines in time. Nevertheless, mice are better protected against enteropathogens such as salmonella, *Escherichia coli* and *Citrobacter rodentium*. Whether SFB are part of the normal human microbiota is still inconclusive despite several studies suggesting their presence. In conclusion, SFB are part of the microbiota of many species, and key players in the maturation of the murine immune system of the gut. They contribute to an enhanced resistance to enteropathogens.

INTRODUCTION

Segmented filamentous bacteria (SFB) are Gram positive, sporeforming, yet non-cultivable bacteria that are related to clostridia. Intestinal spore-forming bacteria with a segmented filamentous appearance that attach to the intestinal wall were originally described in mice (*Hampton* and *Rosario*, 1965) and chickens (*Fuller* and *Turvey*, 1971), but have now been found in a wide range of animals. In mammals, they preferentially adhere to the epithelial cells of the ileum (see Figure 1) and of Peyer's patches, small lymphoid organs involved in antigen sampling from the intestinal lumen.

SFB do not have an official taxonomic name, as they cannot be cultured *in vitro*. Based on their 16S ribosomal RNA sequence, they were given the provisional status *Candidatus* and the genus name Arthromitus until *in vitro* culturing methods become available, and bacteria can be further characterized and added to culture collections. SFB are host specific: ileal bacterial preparations containing SFB did only lead to outgrowth in ex-germfree mice and rats when they were derived from the same species (*Tannock* et al., 1984). Because of this, Arthromitus can be divided into several species,

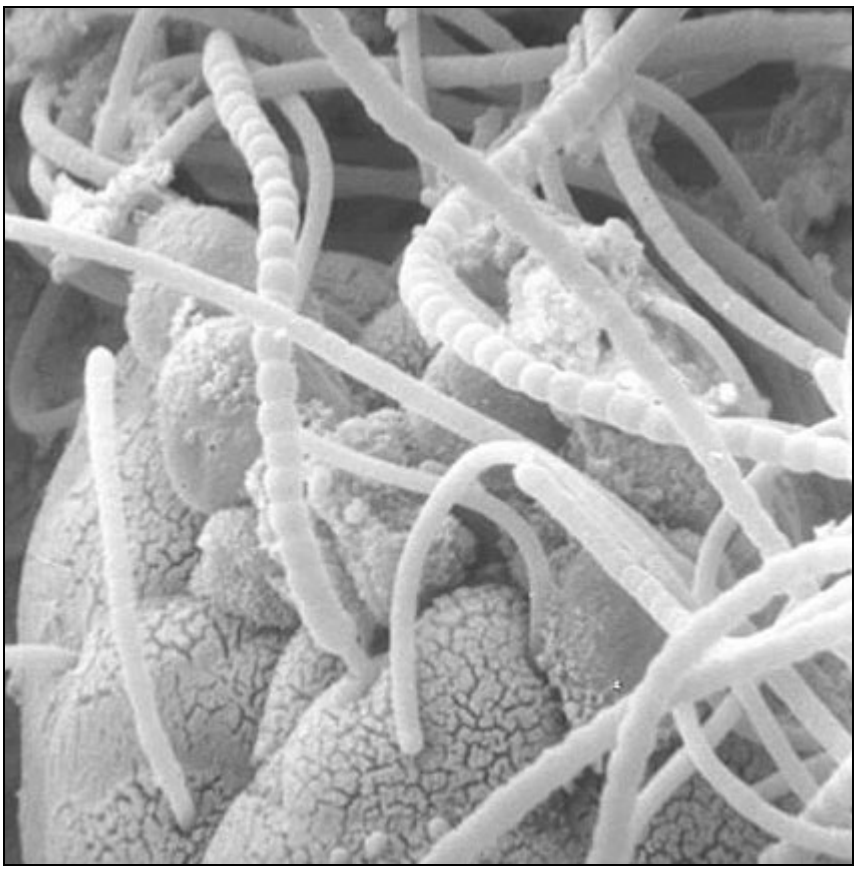

Figure 1: Scanning electron microscopic image of segmented filamentous bacteria in mice. Filaments are attached to epithelial cells without any signs of inflammation. Two morphotypes can be distinguished: smooth filaments and filaments with a beaded appearance.

also since small differences in 16S rRNA sequences are observed between these bacteria in different hosts (*Snel* et al., 1995). The species are designated *Candidatus* Arthromitus muris, *Candidatus* Arthromitus ratti and *Candidatus* Arthromitus galli for SFB from mouse, rat and chicken respectively.

The establishment of monocultures of SFB in ex-germfree mice (*Klaasen* et al., 1991b; *Umesaki* et al., 1995) has greatly facilitated research into these bacteria. Because of the intimate relationship with the host, several studies have suggested that SFB may increase the resistance of the host to infectious diseases. This may be by various mechanisms, including competitive exclusion (*Garland* et al., 1982; *Heczko* et al., 2000), immune activation (*Ivanov* et al., 2009), and stimulation of intestinal transit (*Snel* et al., 1996).

ADHESION AND COMPETITIVE EXCLUSION

SFB adhere to the epithelial cells of the ileum with a special structure described as holdfast (Figure 2). The shape of this holdfast is variable from bean-, tear-

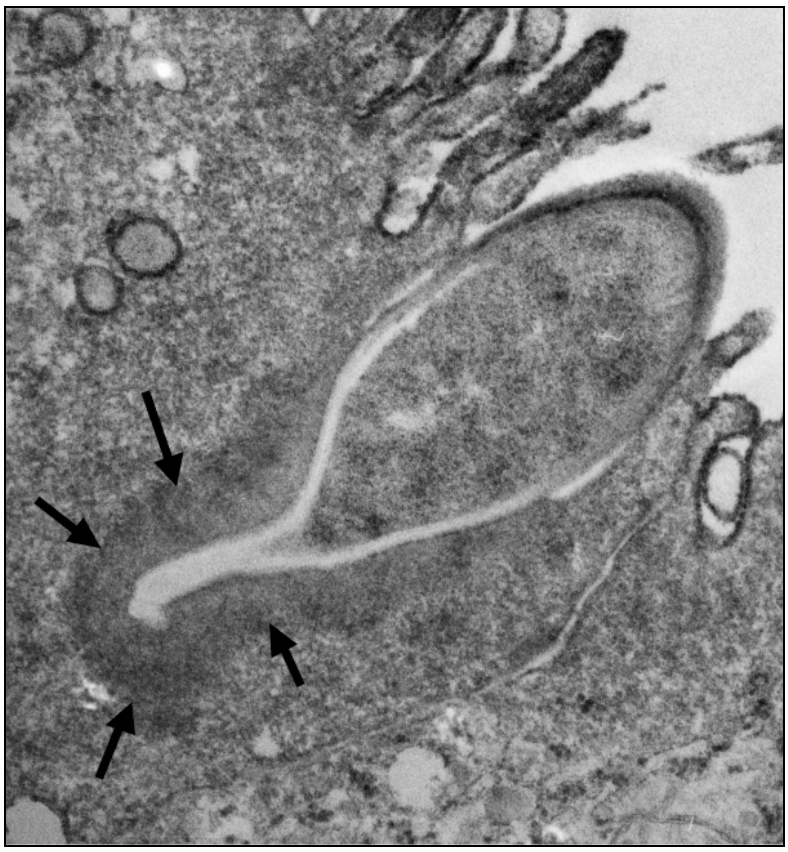

Figure 2: Transmission electron microscopic image of an epithelial cell containing an SFB holdfast. The brush border membrane of the epithelial cell is unaffected. The host cell has responded by accumulation of actin at the site of attachment (arrows).

drop-, to bulb-shaped (*Blumershine* and *Savage*, 1978). Using transmission electron microscopy, it is demonstrated that attachment of SFB causes an invagination of the plasma membrane and displacement of the microvilli at the site of attachment. The host cell responds to adhesion with the accumulation of polymerized actin, similar as seen after attachment of enteropathogenic *Escherichia coli* (*Jepson* et al., 1993), but despite intense association with the gut wall, SFB in mice generally do not possess pathogenic characteristics. In contrast to infection with pathogenic bacteria, the microvilli of the brush border of infected cells remain intact (*Jepson* et al., 1993).

During the weaning phase, peak levels of SFB are observed in the ileum. Colonization of mice starts around weaning at about 3 weeks of age, at which the animals shift their diets from milk to solid food (*Blumershine* and *Savage*, 1978; *Koopman* et al., 1987). Thereafter, levels gradually go down and hardly any adhering SFB are found in adult mice. In immunocompromised animals, the level of adhering SFB in the ileum remains high after weaning (*Snel* et al., 1998). Upon administration of the probiotic *Lactobacillus plantarum* to immunocompromised animals, the expan-

sion of SFB in the ileum of these mice was abolished suggesting direct competition between SFB and *Lactobacillus* (*Fuentes* et al., 2008).

Several electron microscopic studies in mice describe rod-shaped bacteria adhering to the SFB filaments in mice (*Klaasen* et al., 1992a; *Koopman* et al., 1987). The exact nature of these bacteria is unknown, although it is speculated that because of their morphology they may be *Lactobacillus* species (*Koopman* et al., 1987). Similar sub-colonization of SFB by rod-shaped bacteria is found in chickens (unpublished data). Although speculative, the combination of SFB with epibionts may even further strengthen the physical barrier for adherence of pathogenic bacteria. Alternatively, they may represent the direct competition between these bacteria.

ATTACHMENT AND EPITHELIAL RESPONSES

Attachment is not restricted to regular epithelial cells of the ileum: SFB also adhere to the follicle associated epithelium of Peyer's patches in the ileum, specialized lymphoid organs as part of the mucosal immune system (*Jepson* et al., 1993; *Snel* et al., 1998). Within the follicle-associated epithelium, membranous cells (M-cells) are involved in the continuous sampling of antigens from the lumen. Although rarely seen, SFB are capable to adhere to M-cells of mice (*Jepson* et al., 1993; *Meyerholz* et al., 2002), and even extend from an M-cell into intimate association with an intraepithelial mononuclear cell (*Meyerholz* et al., 2002).

It is not surprising that the intimate relationship between SFB and the host results in a strong host response. Global transcriptomic analysis of terminal ileum tissues from healthy adult C3H/HeN germfree mice, conventional mice, and mice conventionalized with whole mouse faecal flora at adult age indicated that 45% of the genes induced by the microbiota could be assigned to immune response pathways (*Gaboriau-Routhiau* et al., 2009). This effect was observed at both 8 and 60 days post-colonization. Epithelial gene expression of a monoculture of SFB was compared to gene expression induced by the probiotic strains *Lactobacillus casei* Shirota and *Bifidobacterium breve* Yakult after 3 days of mono-association of germfree mice (*Shima* et al., 2008). Most pronounced effects were found in the ileum where SFB, far more than the two probiotics, differentially expressed 942 genes (478 more than 2-fold upregulated and 464 more than 2-fold downregulated compared to germfree animals) versus 362 for *Lactobacillus* (183 up and 179 down) and 264 for *Bifidobacterium* (75 up and 189 down). Surprisingly, the overlap in differentially expressed genes by these 3 strains was limited. It was found that, especially in the ileum, many of the SFB-upregulated genes belonged to the functional categories cell communication, defence and immunity, metabolism, and transport.

IMPACT ON THE EPITHELIUM

Changes in gene expression are also reflected in phenotypical changes of the epithelium. In ex-germfree mice associated with SFB, fucosylation of asialo GM1 glycolipid occur in the small intestinal epithelial cells (*Umesaki* et al.,

1995). Using a genomics approach, it was demonstrated that in particular, alpha(1-2) fucosyltransferase was induced in the gut epithelium after mono-association with SFB, but not after mono-association with a *Lactobacillus* or *Bifidobacterium* strain (*Shima* et al., 2008). Another interesting gene induced by SFB is pancreatitis-associated protein (PAP or RegIIIγ). This gene encodes a C-type lectin, and is even found to be induced after mono-association of germfree severe combined immunodeficient (SCID) mice with SFB as an innate response to microbial colonization (*Keilbaugh* et al., 2005). A recent study described antimicrobial activity of the gene product, and suggested a role in gut homeostasis in order to maintain symbiotic host-microbe interactions (*Cash* et al., 2006).

Host epithelial cells in germfree mice are known for their low expression of major histocompatibility complex II (MHC-II) molecules on the apical surface, which is rapidly induced after conventionalization with a complete microbiota. After attachment, SFB can be phagocytised into the epithelial cells of the ileum and intracellularly processed by heterophagy (*Yamauchi* and *Snel*, 2000). Mono-association with SFB results in expression of MHC-II, a phenomenon that is not seen after mono-association with related spore-forming bacteria from the genus Clostridium (*Umesaki* et al., 1995; *Umesaki* et al., 1999).

SFB AND INTESTINAL TRANSIT

One of the observed differences between germfree and conventional mice relates to the slower intestinal transit in the germfree animals that is rapidly increased after the introduction of a normal microbiota (*Abrams* and *Bishop*, 1967). It is suggested that this transit rate contributes to homeostasis of the microbiota in the small bowel (*Caenepeel* et al., 1989). In a study using germfree and mono-associated animals as well as animals with a complex microbiota with or without SFB, it was shown that SFB promotes intestinal transit (*Snel* et al., 1996). Twenty four hours after the addition of non-pathogenic *E. coli* TG1 to these animals, significantly lower numbers of these bacteria were found in the distal part of the small intestine of SFB-containing mice compared to germfree mice and mice associated with *Clostridium inocuum*. This suggests that the influence of SFB on intestinal transit may also contribute to an enhanced resistance to enteropathogens.

IgA INDUCTION

It is known that SFB are abundantly colonizing the epithelium of mice only shortly after weaning (*Davis* and *Savage*, 1974; *Klaasen* et al., 1992a; *Snel* et al., 1998) and for about 10 days after hatching in chicks (*Yamauchi* et al., 1990). During this period, the mucosal immune system is strongly stimulated, resulting in the induction of high levels of IgA plasma cells in the gut lamina propria and secretory IgA in gut secretions (*Klaasen* et al., 1993b). Since SFB colonization is transiently abundant in immunocompetent mice whereas the bacteria persist in athymic nude mice (*Snel* et al., 1998) as well as in IgA-deficient mice (*Suzuki* et al., 2004), it is strongly suggested that it is

the induced immune response that leads to self-limiting colonization levels. A self-limiting response has also been reported for translocation of the Gram-negative bacterium *Morganella morganii*, the number of translocating bacteria begins to drop with the onset of a specific IgA response while colonization of the intestinal lumen is unaffected (*Shroff* et al., 1995).

T-CELL RESPONSES

Colonization of SFB leads to an expansion of αβ-T-cell-receptor bearing intra-epithelial lymphocytes in the gastrointestinal tract (*Umesaki* et al., 1995). This was accompanied by an increase of cells with cytolytic activity. One recent study of the group of Littman demonstrated a unique role of SFB in T cell responses, particularly in respect to Th17 responses since a stimulation of IL17 and IL22 were described (*Ivanov* et al., 2009). Published in the same week, a study from the group of Cerf-Bensussan showed that not only Th17 was stimulated, but that SFB induced a broad spectrum of pro-inflammatory T helper 1 (Th1), Th17, and regulatory T cell responses (*Gaboriau-Routhiau* et al., 2009).

SFB AND ENHANCED RESISTANCE TO INFECTIONS

Because of its non-pathogenic nature and the intimate relationship of SFB with the host, it has been speculated for years that these bacteria might increase host resistance against intestinal infections (*Glick* et al., 1978; *Porvaznik* et al., 1979). It was speculated that the high colonization level of SFB in weaning animals might competitively lead to reduced colonization levels of food-borne pathogens. Indeed, such an effect was observed in rats that were orally infected with *Salmonella enteritidis*: a reduction of surface colonization by these pathogens was found in the presence of SFB (*Garland* et al., 1982). Also rabbits that were infected with enteropathogenic E. coli O103 showed a negative correlation between presence of SFB and pathogen colonization (*Heczko* et al., 2000). In addition, mice mono-associated with SFB that were given *E. coli* had lower levels of coliforms in the small intestine that is likely due to increased intestinal transit (*Snel* et al., 1996). And mice with elevated Th17 activity due to the presence of SFB, and that were infected with *Citrobacter rodentium* had reduced levels of these enteropathogens in their ileum and colon. Last but not least, young adult mice infected with *S. typhimurium* had a prolonged survival when mono-colonized with SFB compared to germfree mice. Such a prolonged survival was not seen in mice mono-associated with *Clostridium innocuum,* and in preweaned mice with SFB but an immune system that is not fully mature (unpublished data).

Altogether, there seems substantial evidence that SFB enhances host resistance. Nevertheless, in another study, using either *S. enteritidis* or *Enterobacter cloacae* as the challenging micro-organisms, the presence of SFB did not lead to significantly reduced translocation of pathogens (*Klaasen* et al., 1992b). Since the animals were only 4-5 wks. of age with likely an immune system that was not fully mature, and since immune status of these

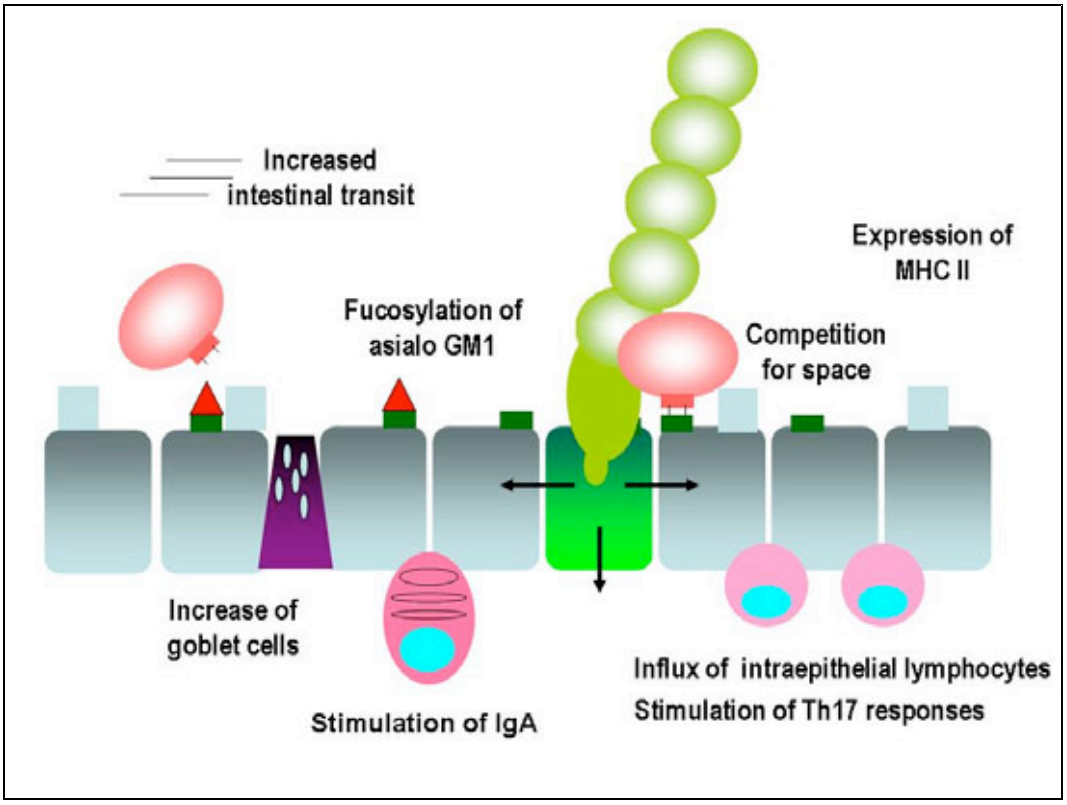

Figure 3: Schematic overview of the defence against enteropathogenic microorganisms. SFB contributes to competitive exclusion by high colonization levels during weaning and stimulates several aspects of immune maturation after weaning.

animals was not monitored, these results from this study are far from conclusive.

The influence of SFB on resistance to enteropathogens because of its high bacterial density during weaning and stimulation of the immune response post-weaning is schematically depicted in Figure 3.

PRESENCE OF SFB IN HUMANS?

The demonstration of SFB in a wide range of host species suggests that these bacteria do not only affect immune maturation of mice but also of other vertebrates, including man. Whether SFB are present in humans is under debate. Although SFB are predominant in mice during weaning, bacteria with a filamentous morphology and adhering capacity have been described in a biopsy from a 67 years old patient undergoing surgery which is not even close to the age of weaning in man (*Klaasen* et al., 1993a). In another study, bacteria were associated with the mucosa of children with celiac disease, with both active and inactive disease, but not with controls (*Forsberg* et al., 2004). These bacteria were described as rod-shaped and were morphologi-

cally not comparable to SFB in mice. In both studies, the nature of these bacteria was not confirmed by e.g. analysis of the 16S rRNA gene sequence, which makes it doubtful that these bacteria were related to *Candidatus* Arthromitus.

The group of MacFarlane at the University of Dundee has studied the microbial composition of a continuous culture system of the human colonic microbiota using a range of phylogenetic DNA probes for fluorescent in situ hybridisation. This continuous culture system was inoculated with faecal material from a 30-year-old male. At various stages of the study, up to 16% of the microbiota reacted with an SFB-specific probe. Unfortunately, the full 16S rRNA sequence was not obtained, and therefore hybridisation may have been non-specific. Especially since so far, large-scale investigations of the human microbiome using P454 pyrosequencing or similar techniques have not revealed sequences with a homology to *Candidatus* Arthromitus.

At present, it is to early to conclude that SFB in humans are not found, especially since diet may have a strong impact on the presence of SFB (*Klaasen* et al., 1991a) and since SFB colonization in humans may be affected by age, like in mice. So far, to our knowledge, systematic studies that investigate the presence of SFB in humans have not been reported.

CONCLUSIONS

Segmented filamentous bacteria are a group of bacteria that are found in the ileum of several animal species, including mammals, birds and fish. Here they have a profound effect on maturation of the immune system, intestinal transit and mucosal epithelium. This does not only affect colonization of SFB itself, but also that of various enteropathogenic microorganisms such as *Salmonella* and *E. coli*. In this way they contribute to enhanced resistance against these enteropathogens. Whether SFB are part of the normal microbiota of humans and play a role in human gut health is still unclear.

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SEGMENTED FILAMENTOUS BACTERIUM: FROM MUTUALISM TO PATHOLOGY?

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INTRODUCTION

The mammalian intestine is colonized by a considerable and complex community of bacteria which develop with their host mutualistic interactions (*Backhed* et al., 2005). To cope with this massive bacterial load, eukaryotic host have evolved a finely tuned intestinal immune barrier that maintains intestinal homeostasis. Studies comparing germfree and gnotobiotic animals have highlighted how the post-natal maturation of this immune barrier is driven and tuned by the microbiota (reviewed in: *Hooper* and *Macpherson*, 2010; *Lee* and *Mazmanian*, 2010). These studies have also shown that individual bacteria may differentially affect the balance between pro- and anti-inflammatory responses in the intestine and beyond (reviewed in: *Cerf-Bensussan* and *Gaboriau-Routhiau*, 2010; *Round* and *Mazmanian*, 2009). Yet, it is now obvious that the impact of the microbiota will depend on the immune status of the host.

INFLUENCE OF SFB ON THE PRO-INFLAMMATORY/REGULATORY BALANCE IN THE GUT

Colonization of germfree mice by complex mouse microbiota results in the simultaneous induction of pro-inflammatory and regulatory responses (*Gaboriau-Routhiau* et al., 2009; *Geuking* et al., 2011). Recent works have stressed the important regulatory function of Foxp3+ regulatory T (Treg) cells in the colonic mucosa to limit the expansion of inflammatory T cell subsets, possibly via the production of IL-10 (*Atarashi* et al., 2011; *Geuking* et al., 2011). Some strains of bacteria, notably *Bacteroides fragilis*, emerged as prominent inducers of intestinal Treg cells and IL-10 secretion. Accordingly, colonisation by the latter bacteria could prevent the development of inflammation in mouse models of colitis (*Mazmanian* et al., 2008; *Round* et al., 2011; *Round* and *Mazmanian*, 2010). In contrast, other strains, the prototype of which is Segmented Filamentous Bacteria (SFB) can exert a much broader impact on the gut immune system.

SFB are Clostridia-related bacteria which settle in the rodent intestine at the time of weaning and tightly adhere to ileal epithelial cells (and to Peyer's patches) during the first weeks of colonization. As a possible consequence of their adherence to the ileal mucosa and Peyer's patches, SFB can

strongly stimulate postnatal maturation of mouse intestinal immune responses. SFB efficiently activate the expansion of intestinal IgA-producing plasma cells and the recruitment of intra-epithelial lymphocytes (*Klaasen* et al., 1993; *Talham* et al., 1999; *Umesaki* et al., 1995). SFB also induce the differentiation of a broad spectrum of innate and of pro- and anti-inflammatory T cell responses (*Gaboriau-Routhiau* et al., 2009). Notably, mice colonized by a microbiota that does not contain SFB, lack intestinal Th17 cells (*Gaboriau-Routhiau* et al., 2009; *Ivanov* et al., 2009) and cannot control infection by *Citrobacter rodentium* (*Ivanov* et al., 2009). As highlighted by J. Snel in this issue, colonization by SFB can thus benefit to the host by stimulating an efficient intestinal barrier which opposes invasion by enteropathogens. Yet, the beneficial pro-inflammatory effect of SFB observed in immune-competent hosts is possible only when counterbalanced by a regulatory response that maintains "physiological inflammation" (or controlled inflammation) critical for intestinal homeostasis. Such benefit is lost in hosts with impaired immunoregulation. Thus, in severe combined immunodeficient (SCID) mice transferred with naive CD4+ T cells (a model where regulatory T cells are absent), colonization by SFB promoted the onset of colitis (*Stepankova* et al., 2007). The deleterious role of SFB was however only observed when mice were simultaneously colonized by a specific pathogen free (SPF) microbiota, underscoring the fact that SFB is not *per se* a pathogen and that additional signals from the microbiota are necessary to trigger deleterious intestinal inflammation in this model.

IMPACT OF THE MICROBIOTA AND OF SFB OUTSIDE THE GUT

There is ample evidence that the intestinal microbiota can influence the peripheral immune system. Thus, comparison between conventional and germfree mice have shown that spleens of germfree animals contain fewer and smaller germinal centres (*Macpherson* and *Harris*, 2004) and a decreased number of CD4+ T cells with a skewed Th2-type profile (*Dobber* et al., 1992; *Mazmanian* et al., 2005). Yet, specific responses to bacterial antigens, as reflected by specific serum IgG or specific proliferative responses of spleen CD4+ T cells against bacterial antigens, were only observed in hosts with defective intestinal immune barrier and were associated with increased bacterial translocation into the spleen (*Konrad* et al., 2006; *Macpherson* and *Uhr*, 2004; *Slack* et al., 2009). Altogether, these results suggest that, in immunocompetent hosts, bacterial products (but not bacteria) may cross the intestinal barrier and exert an adjuvant effect on peripheral responses. Accordingly, peptidoglycan was shown to reach the bloodstream and bone marrow, and activate bactericidal activity of neutrophils through stimulation of their innate immune receptors (*Clarke* et al., 2010). Translocation of lipopolysaccharide, favoured by lipid-rich diet, has been associated with inflammation in adipose tissue and onset of metabolic disorders (*Cani* et al., 2008). Along the same line, oral administration of the capsular polysaccharide A of *Bacteroides fragilis* could recapitulate the effect of colonization of germfree mice by this bacterium, i.e. increase the number of T cells in the spleen and correct the Th1/Th2 imbalance (*Mazmanian* et al., 2005).

The role of the microbiota on systemic immunity has also been recently addressed in various experimental disease models in either competent or immunocompromised hosts. This role varies according to models (reviewed in: *Cerf-Bensussan* and *Gaboriau-Routhiau*, 2010). Several severe models of autoimmunity affecting central tolerance developed independently of the microbiota. In contrast, a protective effect of the microbiota was observed in the models of collagen-induced arthritis (*Breban* et al., 1993) and of non-obese diabetic (NOD) mice-associated type 1 diabetes (*Wen* et al., 2008). Interestingly, reduced sensitivity to type 1 diabetes was observed in female NOD mice only when SFB was present in their faecal microbiota, and protection was ascribed to the induction of IL-17-producing T cells in the small intestinal lamina propria of SFB-positive females (*Kriegel* et al., 2011). Yet, the reduced sensitivity to diabetes observed in males was neither associated with SFB detection nor with lamina propria Th17 response, suggesting that complex host-microbiota interactions control the onset of diabetes in NOD mice.

Contrasting with these data, the microbiota promoted the onset of autoimmunity in other mouse models. Thus, inflammatory arthritis in transgenic K/BxN mice and experimental autoimmune encephalomyelitis (EAE) induced by myelin-derived proteins were attenuated in germfree mice compared to mice colonized with SPF microbiota (*Lee* et al., 2010; *Wu* et al., 2010). In both models, mono-colonization with SFB could largely recapitulate the impact of the SPF microbiota. In EAE, the deleterious impact of SFB was associated with a strong adjuvant effect on the immune system, as illustrated by a simultaneous increase in pro-inflammatory (IL-17, IFNγ) and regulatory T cell responses, notably in the spinal cord (*Lee* et al., 2010). In K/BxN mice, the arthritis results from an uncontrolled pro-inflammatory Th17 response in the periphery, that drives the production of auto-antibodies and deposition of immune complexes in the joints (*Wu* et al., 2010). SFB, alike an SPF microbiota, stimulated a strong increase in the number of spleen Th17 cells, which may partly derived from the intestinal compartment (*Wu* et al., 2010). This model however precludes the efficient induction of regulatory T cells. Altogether, these data indicate that the microbiota, and notably SFB, can influence the onset of autoimmune diseases outside the gut. The impact of the microbiota and of SFB, however, largely depends on the host immune status and/or on the mechanisms of the diseases.

CONCLUSION

In the complex cross-talk between the host and its microbiota some bacteria, such as SFB, have emerged as key drivers of the physiological inflammation that maintains intestinal homeostasis. However, the equilibrium between the host and the microbiota is fragile and SFB can become a risk factor for disease development when host immunoregulatory mechanisms are impaired. It will be interesting to identify an SFB-like bacterium in the human microbiota and examine how this bacterium might contribute to human health and disease.

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THE MICROBIOTA IN THE DEVELOPMENT OF COLITIS DUE TO *CLOSTRIDIUM DIFFICILE* INFECTION

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SUMMARY

One of the unexpected side effects of antibiotic treatment is the development of colitis. Ranging in severity from nuisance diarrhoea to fulminant pseudomembranous colitis leading to toxic megacolon and perforation, antibiotic-associated colitis is thought to arise from changes in the community structure of the indigenous gut microbiota. The most severe cases of antibiotic-associated colitis are generally due to infection with the toxin-producing bacterium *Clostridium difficile*. Owing to advances in the culture-independent analysis of complex microbial communities that were developed by researchers studying environmental bacterial consortia, we are now in the position to understand how antibiotics alter the indigenous gut microbiota and how these changes lead to susceptibility to *C. difficile* infection. In this review, we will summarize the early work investigating the interaction between *C. difficile* and the gut microbiota and highlight more recent studies using molecular microbial ecology techniques to study *C. difficile* infection humans and animal models of disease.

INTRODUCTION

The development of antibiotics can arguably be regarded as one of the major medical advances of the 20th century. However, as history has shown time and time again technological advances can lead to unintended and unforeseeable consequences. With respect to antibiotics, unintentional alteration of the indigenous microbiota of the host through their broad administration has had significant health effects both acute and chronic. In this review we will discuss one of the earliest observed side effects of antibiotic administration, the development of diarrhoea and colitis.

The development of antibiotics can be viewed as an arms race between researchers in the pharmaceutical industry who are developing novel drugs, often with broader and broader spectrum of activity, and the microorganisms that develop ever-expanding mechanisms of resistance. As the spectrum of activity of the drugs grew in an attempt to circumvent resistance and to allow more effective empiric treatment of life-threatening infections, novel side effects were noted with increasing frequency among patients receiving these treatments. In particular, treatment with the broad-spectrum antibi-

otic clindamycin was associated with a clinical condition that became known as "clindamycin colitis" (*Tedesco* et al., 1974a,b). Patients receiving clindamycin would develop diarrhoea, which in milder cases was simply regarded as a "nuisance" but a proportion of patients would have progressive disease with severe abdominal pain, systemic signs and symptoms and in a minority of cases would progress to toxic megacolon, intestinal perforation and death.

FULFILLING KOCH'S POSTULATES FOR *CLOSTRIDIUM DIFFICILE*

During the 1970s several groups sought to determine the cause of "clindamycin colitis". *Staphylococcus aureus* was one of the first organisms that was implicated as a potential infectious agent causing antibiotic-associated colitis (*Khan* and *Hall*, 1966). Part of this assertion stemmed from the observation that the administration of vancomycin would be an effective treatment for clindamycin colitis. However, the work of two major groups helped fulfil Koch's postulates implicating *Clostridium difficile* as the causative agent of antibiotic associated colitis. These two groups, one led by John Bartlett at Harvard and the other led by Robert Fekety at the University of Michigan, utilized a hamster model of clindamycin-associated colitis (*Bartlett* et al., 1977; *Lusk* et al., 1978). These investigators treated hamsters with clindamycin and subsequently experimentally challenged them with toxin-producing *C. difficile*. This would result in the rapid development of colitis and the subsequent death of the animals thus fulfilling Koch's postulates. These initial animal experiments led to a number of important follow-up studies including the identification of the two major toxins produced by *C. difficile*, establishment of vancomycin and metronidazole as relatively effective treatments for *C. difficile* infection (CDI), the development of diagnostic tests based on the detection of *C. difficile* toxins and the observation that multiple antibiotics could result in subsequent CDI. Thus, in the early 1980s it was felt that much of the major work on *C. difficile* had been completed and much of the attention of infectious disease practitioners and researchers was diverted to other areas, most notably the emerging epidemic of HIV. A number of groups continued to investigate the molecular mechanisms underlying *C. difficile* pathogenesis, most often focused on the biology of the *C. difficile* toxins (*Voth* and *Ballard*, 2005). Renewed interest in this infection came with the recognition of the emergence of a second epidemic of CDI in the early 21st century. This second wave of illness was associated with a previously unrecognized hyperendemic strain of *C. difficile* (denoted as NAP1/BI/027 on the basis of PFGE, REA typing and ribotyping) and increasing severity of disease (*McDonald* et al., 2005; *Pepin* et al., 2004). Simultaneous with the recognition of this new epidemic of CDI, the growing application of research approaches from the field of environmental microbial ecology to host-associated microbial communities as allowed a greater understanding of the pathogenesis of *C. difficile* infection related to changes in the indigenous microbiota secondary to antibiotic administration. We will now review some of the concepts related to microbial ecology and how the indigenous microbiota can mediate "colonization resistance" against pathogenic microbes.

THE INDIGENOUS GUT MICROBIOTA

The indigenous human microbiota includes at least ten thousand different kinds of microbes with a total genetic complexity (the microbiome) that exceeds twenty billion base pairs and codes for more than forty million genes (*Dethlefsen* et al., 2007). Differences in nutrition, host physiology, immunity, state of health and other ecological stressors lead to significantly different microbial population structures and their collective metagenome within and between human hosts. The goal of the Human Microbiome Project is to understand how these complex, dynamic consortia shape human health and well-being (*Turnbaugh* et al., 2007).

The complex community of microbes that inhabits the mammalian gut represents an assemblage of organisms that exists in a balanced symbiosis with its host (*Ley* et al., 2006). The metabolism of the gut microbiota participates in the catabolism of ingested nutrients and also produces a wide variety of ligands and antigens that can interface with the host metabolism and immune system. Therefore, alterations in the community structure of this microbial community can have implications on the homeostasis of the host. Changes in the gut microbiome correlate with obesity, inflammatory bowel disease and antibiotic-associated diarrhoea. These pathologic conditions likely arise via changes in the metabolic activity of the altered microbial community or alterations in the interaction of the microbiome with the host immune system.

THE GUT MICROBIOTA AND COLONIZATION RESISTANCE

The term colonization resistance was coined to refer to the ability of a previously established gut microbial community to resist invasion by an additional microbe (*Freter*, 1962; *Hentges* and *Freter*, 1962; *Vollaard* and *Clasener*, 1994). Although this initially applied to pathogenic microbes, the concept was derived from concepts of community robustness applied to "classical" ecologic systems (for example grasslands and lakes) and thus could be applied to any invading microbe. Current dogma holds that the normal indigenous microbiota is not permissive for colonization of *C. difficile* (*Wilson*, 1993). In the minority of cases where normal individuals are colonized by *C. difficile* without overt clinical disease, it is further hypothesized that the normal indigenous microbiota can at least limit the production of toxin, perhaps by directly interfering with toxin production or limiting the population size of *C. difficile* and preventing significant amounts of toxin from accumulating in the gut. According to this model, disruption of the indigenous microbiota by antibiotics leads to a loss of colonization resistance, making the gut vulnerable to colonization by exogenous *C. difficile* spores or, in previously colonized patients, expansion and toxin production. As noted above, treatment of Syrian hamsters with antibiotics induces a lethal, haemorrhagic colitis (*Bartlett* et al., 1978, 2004). When initially described, this was found to be due to expansion of indigenous *C. difficile* and an increase in toxin production. Wilson and colleagues provided evidence for the ability of the normal gut microbiota to inhibit *C. difficile* by demonstrating that administration of normal caecal homogenates would decrease the num-

ber of viable *C. difficile* and prevent colitis in antibiotic-challenged hamsters (*Wilson* et al., 1981). Subsequent studies in a variety of model systems started to precisely define which members of the indigenous microbiota played a role in mediating colonization resistance. However, the complexity of the gut microbial community and the limitations in the culture-dependent methods that were utilized at the time prevented the performance of more than descriptive studies. The recent development of culture-independent methods of following complex microbial communities and the advent of genomic technologies allows current investigators to revisit hypothesis-driven studies of colonization resistance.

Initial studies of microbial diversity were dependent on the ability to isolate and culture organisms on artificial media. The inability to culture a majority of the microbes present in complex environmental communities has lead to the development of a variety of non culture-based methods for assessing microbial diversity. Over thirty years ago, Woese pioneered the use of rRNA sequence comparisons for charting the evolutionary history of microbes (*Sogin* et al., 1971; *Woese* and *Fox*, 1977; *Woese* et al., 1974). With the introduction of improved sequencing technology, Pace developed culture-independent, molecular tools for assessing the diversity and ecology of microorganisms (*Pace*, 1997; *Pace* et al., 1985). The number of microbial phyla increased from Woese's description of a dozen bacterial lineages to more than 100 major phyla, most of which do not include cultured representatives (*Ley* et al., 2006). Sequence analysis of PCR amplicons for rRNA genes is now the "gold standard" for assessing species richness in microbial communities. Databases contain more than 500,000 rRNA sequences that correspond to phylotypes from diverse microbes*.

Based on surveys using rRNA gene sequences as proxies for the presence of a microbe in a sample, microbial diversity is at least 100-1000 times greater than estimates based upon cultivation-dependent surveys (*Pace*, 1997). Yet these "first generation" molecular assays, involving the amplification, cloning and sequencing16S rRNA-encoding genes, have only captured a fraction of microbial diversity and they rarely provide estimates of relative abundance for differ- ent kinds of microbes or operational taxonomic units (OTUs) (*Pedros-Alio*, 2006, 2007). The occurrence and distribution of the low-abundance taxa remain under-sampled and uncharted (*Pedros-Alio*, 2007). The most extensive molecular surveys of intestinal microbial flora report 13,000-18,000 rRNA gene sequences from humans (*Eckburg* et al., 2005; *Ley* et al., 2006), with one study noting great differences between microbial communities of mucosal surfaces and faeces. Several smaller surveys of gut flora describe correlations with disease states (*Bibiloni* et al., 2006; *Eckburg* and *Relman*, 2007; *Hopkins* et al., 2001; *Zoetendal* et al., 2002), the establishment of gut flora (*Favier* et al., 2003; *Magne* et al., 2006), and effects of antibiotics (*Shoemaker* et al., 2001), etc. However, the limited sampling effort for these investigations is not sufficient to

*http://bioinformatics.psb.ugent.be/webtools/rRNA/
http://www.arb-silva.de
http://ncbi.nlm.nih.gov/
http://greengenes.lbl.gov/cgi-bin/nph-index.cgi)

comprehensively describe the diversity of the human microbiome, and none have addressed the influence of low abundance microbial populations on human health.

Emerging population structure studies of microbial communities from marine environments (*Huber* et al., 2007), soils (*Ashby* et al., 2007; *Roesch* et al., 2007), and the human gut (*Eckburg* et al., 2005; *Ley* et al., 2006) reveal complex diversity patterns where a few dominant phylotypes mask the presence of many thousands of different kinds of lower abundance microbes, many of which represent completely novel OTUs. These studies suggest that even the largest of published metagenomic investigations will not represent the full extent of microbial diversity unless they incorporate normalization procedures to insure the representation of all abundance classes. Implicit in this strategy is the requirement to determine the full complexity of human microbiota or to reduce the population complexity through new cultivation strategies. The descriptions of community complexity must include estimates of the number of different kinds of microbial taxa (richness) and their relative abundance (evenness) for abundant, moderately abundant and rare microbial populations including members of the "rare biosphere". In response to the expense and limited throughput of traditional non culture-based surveys of 16S rRNA-encoding gene diversity, "next generation" technologies (detailed below) have been developed that leverage the power of the newest high-throughput DNA sequencing technologies to provide insight into the rare biosphere. Traditional clone libraries and these next-generation technologies will be employed synergistically in the current proposal to provide a much more comprehensive view of the diversity of the gut microbiota.

MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT UNDER THE INFLUENCE OF ANTIBIOTICS

Human Studies

Although the focus of this discussion is on *C. difficile* , it should be noted that infection with *C. difficile* is estimated to be responsible for only 25% of all cases of antibiotic-associated diarrhoea, although it is generally the cause of the most severe forms of the disease. In the remainder of cases alteration of the normal indigenous microbiota is felt to interfere with the digestion of complex carbohydrates resulting in an osmotic diarrhoea. In one of our initial studies (*Young* and *Schmidt*, 2004) to use culture-independent analysis to profile the gut microbiota we followed changes in the faecal bacterial community of an individual who developed diarrhoea on amoxicillin/clavulanic acid. DNA was purified from faecal samples and the diversity of the bacterial microbiota determined by amplification using broad-range PCR primers that target conserved regions of the 16S rRNA-encoding gene. Our analysis demonstrated that antibiotic administration resulted in dramatic shifts in the composition of the faecal microbiota. Of note there was a marked decrease in members of the family Clostridiaceae. These organisms are known to ferment complex carbohydrates to short chain fatty acids including butyrate, which is the preferred energy source for colonic enterocytes (*Topping* and *Clifton*, 2001; *Wong* et al., 2006). Interestingly, two weeks after the discontinuation of antibiotics the community structure returned largely to baseline.

In a subsequent study we profiled the bacterial microbiota from the faeces of patients diagnosed with antibiotic-associated diarrhoea due to *C. difficile* (*Chang* et al., 2008). In particular, we compared patients who presented with an initial episode of *C. difficile* diarrhoea to patients with recurrent disease. We noted that whereas patients with an initial episode of *C. difficile*-associated diarrhoea had a faecal microbiota with similar diversity to matched controls, the microbiota from patients with recurrent disease exhibited significantly decreased the diversity. These findings provided support for the hypothesis that recurrent *C. difficile*-associated diarrhoea is in at least partly due to persistent abnormalities in the gut microbiota that lead to diminished colonization resistance. Furthermore these findings suggest that the use of faecal transplants for recurrent *C. difficile* infection (*Aas* et al., 2003; *Tvede* and *Rask-Madsen*, 1989) is successful due to restoration of the normal diversity of the gut microbiota and restoration of colonization resistance. A recent study has confirmed this supposition by demonstrating that faecal transplants do have lasting effects on the gut microbiota of the recipient (*Khoruts* et al., 2010).

Other groups have studied the effects of antibiotic administration on the indigenous gut microbiota of healthy volunteers. In general, antibiotic administration has a profound affect on the community structure of the gut microbiota over the short term. There is variability as to the long-term effects of such antibiotic administration. For example, in our initial study we noted that two weeks after the administration of amoxicillin/clavulanate was discontinued, microbial diversity largely returned to the baseline state (*Young* and *Schmidt*, 2004). In another study studying the administration of ciprofloxacin in healthy volunteers, patients had variable recoveries (*Dethlefsen* et al., 2008; *Dethlefsen* and *Relman*, 2011). In some cases, recovery was largely complete following cessation of antibiotics but in other cases patients had long-term changes in their microbiota thus indicating that individual variation in the baseline microbiota and potentially other host factors could influence the response to antibiotic perturbation.

Animal Studies

The above discussion of studies related to antibiotic perturbation in human patients and healthy volunteers suggests that the significant baseline variability of the indigenous microbiota and host genetics has a strong influence on the subsequent behaviour of the microbial community during times of ecologic stress. In an attempt to limit individual-to-individual variation we have turned to inbred murine models to determine if there are reproducible patterns that can be discerned with regards to the response of the gut community to antibiotic perturbation.

In one experiment we utilized a pyrosequencing-based approach that targeted the V6 region of small subunit rRNAs (*Sogin* et al., 2006). The strategy generated over two million V6 tag sequences from bulk DNA extracted from mouse gut tissue. Tag analysis showed that microbial communities of individual co-housed control animals had a remarkably similar structure. The minimal animal-to-animal variation indicates that the gut microbiota in genetically identical animals with the same input microbiota (as all were littermates) follow “assembly rules” that govern the climax structure of the microbial community. This finding is important as the existence of assembly rules implies that variations in commu-

nity structure can provide insight into underlying changes in the gut ecologic environment. For example variables such as host genotype that influences the immunologic state could be reflected in a particular community structure.

Next, we treated inbred mice with two different antibiotic regimens. The first involved a combination of amoxicillin, metronidazole and bismuth and the second was administration of the broad-spectrum cephalosporin antibiotic cefoperazone. Both treatment regimens resulted in significant but distinct shifts in the baseline microbiota (*Antonopoulos* et al., 2009). However, the recovery of the community from these two different stressors was quite distinct. The gut microbiota in animals treated with the combination of amoxicillin, metronidazole and bismuth largely recovered its baseline community structure following the discontinuation of the antibiotics. However, the community in animals treated with cefoperazone remained quite distinct from the baseline even six weeks after the continuation of the drug. The overall biomass of bacteria, which was markedly decreased immediately following the administration of cefoperazone, returned to baseline levels. However, overall diversity of the community remained markedly depressed. These results imply that cefoperazone treatment represents a more severe ecologic stress that AMB administration, a result that could be inferred from the known antimicrobial spectrum of the treatments with cefoperazone having a much broader spectrum. These data indicate that short-term antibiotic administration can have significant effects on the indigenous gut microbiota and these effects may persist long after the discontinuation of the drug.

Murine Models of CDI and the Microbiome

As noted above, Koch's postulates for *C. difficile* and antibiotic-associated colitis were first fulfilled using a hamster model of infection (*Bartlett* et al., 1977). To date this particular animal model has been the most extensively used to study the pathogenesis of *C. difficile*. However, intestinal disease induced in the hamster model is invariably severe, resulting in death within three days after experimental infection. Furthermore, there are limited reagents to study host responses in the setting of hamster infection. For these reasons and others a number of investigators have attempted to develop murine models of *C. difficile* infection. Although mice can be colonized with *C. difficile*, minimal intestinal pathology is encountered which limits the use of this model to study pathogenesis. Germfree mice can be challenged with *C. difficile* and this does result in severe colitis (*Onderdonk* et al., 1980; *Wilson* and *Freter*, 1986) however, this model does not mimic the human situation where a complex indigenous microbiota is present. As such, this system does not let us understand the mechanisms by which the indigenous microbiota mediate colonization resistance, but can provide some insight in to the role of *C. difficile* virulence factors in the disease process.

An important advance in the development of murine models of *C. difficile* infection was recently reported by Chen and colleagues (*Chen* et al., 2008). This group reported that a three day pre-treatment with a cocktail of a five antibiotics (gentamicin, kanamycin, colistin, metronidazole and vancomycin) followed by a two day "washout" period without antibiotics results in a condition where a single dose of

clindamycin followed 24 hours later by experimental infection with *C. difficile* results in the development of colitis. Several aspects of this model make it much more analogous to the situation encountered in human patients compared to the hamster model. Titration of the challenge dose of *C. difficile* can be accomplished to modulate disease severity. In the published study a dose of 10^4 CFU *C. difficile* resulted in a 50% mortality rate. Treatment of *C. difficile*-infected mice with vancomycin, one of the standard treatments for human disease, can prevent mortality although discontinuation of the vancomycin results in disease recurrence. Therefore this model can also be used to model important aspect of human infection with *C. difficile*.

We have recently continued to develop this model of CDI and have used it to study the role of the microbiome in disease pathogenesis (*Reeves* et al., 2011). When we utilized the model as originally described with the cocktail of five antibiotics followed by a single dose of clindamycin prior to experimental infection with *C. difficile* we noted that both treatments were required. If animals received only the five antibiotic cocktail or only a single dose of clindamycin they remained resistant to infection with *C. difficile*, neither being colonized nor developing any clinical or histopathologic evidence of colitis. We compared the microbial ecology of these different antibiotic treatments and found a correlation between loss of members of the phylum Firmicutes and susceptibility to *C. difficile* infection. It is notable that most of the bacteria whose loss was associated with susceptibility to CDI have been postulated to be efficient producers of short chain fatty acids. As previously discussed SCFA is felt to be important for gut homeostasis. We also noted that animals treated with the antibiotic cocktail and clindamycin prior to *C. difficile* infection could follow one of two trajectories. As noted in the original description of the model a certain number of animals would quickly succumb to infection while others appeared to be able to exert some level of control and would not develop clinically severe disease although still were colonized and had histopathologic colitis. When we compared the microbial communities from healthy versus sick animals we noted that animals that appear to be controlling the infection had return of the SCFA-producing Fimicutes while the gut communities in the animals that were clinically ill remained deficient in these organisms.

We also determined if cefoperazone administration, which as noted above could have long-lasting effects on the murine gut microbiota, also led to susceptibility to *C. difficile* infection. We found that cefoperazone alone could lead to a loss of colonization resistance, which was more profound than that induced by the antibiotic cocktail and clindamycin. Animals treated with a course of cefoperazone and then experimentally challenged with *C. difficile* uniformly succumbed to infection within 2 to 3 days. These animals had much higher pathogen levels than animals treated with the antibiotic cocktail clindamycin prior to challenge. Once again, there was a significant loss of the SCFA-producing Firmicutes in cefoperazone treated animals.

CONCLUSIONS

The development of colitis due to *C. difficile* infection following antibiotic

administration represents a disease whose pathogenic mechanism is at the intersection of two schools of thought. Much of the advances we have made in understanding infectious diseases in the past century have been influenced by the theories developed by Robert Koch. We generally think of microbial diseases being the result of infection with a single etiologic agent. Clearly, *C. difficile* infection following antibiotics falls into a disease where a single etiologic agent has been shown to be responsible. However, it is also clear that changes in the complex indigenous microbial community of the gut are also a key feature of the pathogenesis of CDI. We are in the initial stages of understanding what specific changes in the gut community lead to susceptibility to *C. difficile*. Future work defining what functional impact specific changes in microbiota structure have is needed to further understand how alteration of the microbiome leads to CDI. This will also permit us to understand diseases where no single infectious agent can be found and thus pathogenesis is due solely to changes in complex communities. This newer school of thought on the pathogenesis of microbial-associated diseases is only in its infancy and is likely to lead to novel methods for the prevention and treatment of diseases as diverse as obesity, diabetes and inflammatory bowel disease.

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CLOSTRIDIUM DIFFICILE: AN OLD FRIEND IN OUR GI-TRACT

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SUMMARY

Clostridium difficile is an anaerobic, sporeforming Gram-positive rod commonly occurring in the environment as well as in the gastro-intestinal tract of most mammals.It may produce at least 3 different biologically active substances, i.e. toxin A and B and a motility enhancing substance,

In humans it is established in nearly all infants and in this age group the presence of toxin-producing strain(s) is seldom associated with clinical symptoms. Detection-problems may be a major reason for an assumption that *Cl. difficile* is more rare in intestinal microbiota from adults. Data from individuals receiving one dose of antibiotics before undergoing minor elective surgery indicate that around half of adults may harbour *Cl. difficile* in their intestinal microbiota.

In the following some evidence for an assumption that *Cl. difficile* is involved in establishment and maintenance of intestinal motility is summarized. Experiments, carried out in mono-associated rats, showed that the presence of toxins caused a dramatic, but temporary reduction in mitotic activity without causing clinical signs or symptoms.

It is hypothesized that the presence of secondary "troublemakers" may be needed for the establishment of clinical symptoms. In the future, more efforts should be allocated to questions around identifying the possible "troublemakers".

THE MICROBE

Clostridium difficile is a strictly anaerobic Gram-positive rod with elongated spores of about the same width as the rod itself. When grown on blood agar, the colonies are irregular, rough and non-haemolytic. It ferments glucose, mannose and mannitol, but not galactose, lactose, saccharose, raffinose and inulin. It is indole-negative, but some strains may produce hydrogen sulphide. Some strains produce a filterable, thermo-labile toxin which induces local oedema and convulsion in guinea pigs. "The toxin is lethal on injection into dogs, rats, guinea pigs, rabbits and pigeons, but has no effect by mouth in the rat, guinea-pig and dog". This information is taken from the leading book in medical microbiology 35 years ago (*Topley and Wilson's principles of bacteriology, virology and Immunity*, 1975) in which it was placed in a subchapter, entitled "Notes on less important strains". Basically, the infor-

Table 1: Incidence in faeces of *Cl. difficile* and its cytotoxin after one dose of a prophylactic antibiotic to patients undergoing minor elective surgery

Antibiotic	Dose	Incidence of *Cl. difficile* (%)	Toxigenic strains (%)
Mezlocillin	2 g	3	100
Cefoperazone	2 g	44	57

"No patient experienced diarrhoea" (*Privitera* et al., 1991)

mation is still true, but much more has been added since then.

Clostridium difficile was firstly isolated from the stools of healthy newborns by Hall and O'Toole as early as in 1935 (*Hall* and *O'Toole*, 1935). They referred to the organism as *Bacillus difficile* because of the difficulties that they encountered during the isolation of the organism. Shortly afterwards, the isolates were correctly renamed to *Clostridium difficile*. Interestingly, they proposed that the strain has some biological activity because filtrates produce muscular activity in some animal experiments.

Cl. difficile - either as spores or vegetative forms - is widely distributed. It has been isolated from soil, sand and intestinal contents of most mammalian species. In hospitals, spores are always found when thoroughly investigated for. Thus, attempts to totally protect an individual from being exposed to *Cl. difficile* will usually fail.

Over the years, many approaches have been used for the isolation of *Cl. difficile.* The most commonly used medium for isolation of *Cl. difficile* from stools is the cycloserine-cefoxitin-fructose-egg-yolk medium developed in 1979 (*George* et al., 1979). This medium serves as a selective and differential medium for *Cl. difficile* and is reported to detect as few as 2000 organism in a total number of more than a billion other organisms per gram wet weight of faeces. It is uncertain whether molecular microbiological methods can increase the sensitivity of detection.

In general, the carrier-rate of *Cl. difficile* in healthy adults is reported to be up to 5%.However, even a negative cultivation result does not exclude the presence of Cl. *difficile* in the large intestine. As is indicted in a study from Italy (*Privitera* et al., 1991) the carrier rate may be higher (Table 1).

In infants, the carrier rate is much higher, in some places up to nearly 100%. In fact, it has been estimated that "50% or higher of infants are colonized with toxigenic *Cl. difficile* and are asymptomatic" (*Lyerly* et al., 1988). This strongly indicates that *Cl. difficile* may have a physiological role to play.

It has been known for a long time that some strains of *Cl. difficile* might produce biologically active substances, as toxin A, toxin B, and substance(s) with motility enhancing properties. Some biological effects of these substances will be highlighted in the following paragraphs.

CLOSTRIDIUM DIFFICILE AND INTESTINAL MOTILITY

Over the years, the results of studies in different animal species have described a considerable enlargement of the caecum in germfree animals in comparison with conventionally reared animals (Figure 1). In the 1960-ties, it was also

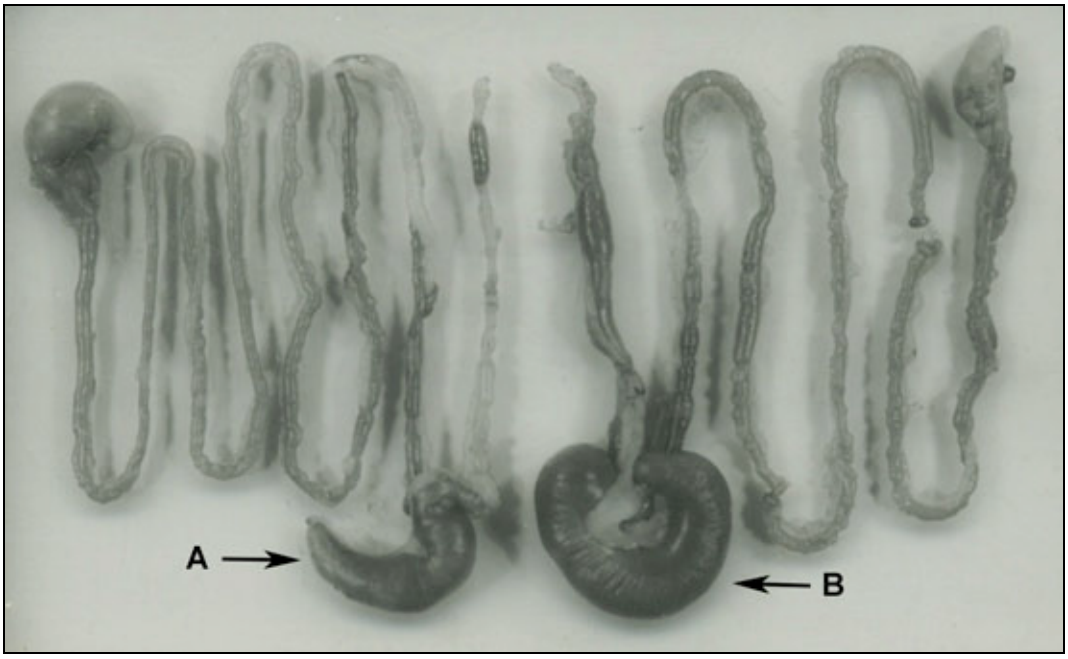

Figure 1: Intestinal tract of a conventional and a germfree rat of the same age. Note the difference in caecum-size between conventional (A) and germfree (B).

well established that when germfree animals were given intestinal content from their conventional counterparts, their caecum-size was gradually reduced, At that time, however, neither the mechanisms behind, nor the microbial species involved were known. It was also an everyday experience for those working with germfree and conventional animals, that spontaneous contractions of the large intestine were commonly observed in conventional animals, but that they were absent in their germfree counterparts. Similarly, the intestinal wall was more reactive to mechanical stimuli in conventional than in germfree animals. These observations created the background for the two following series of experiments:

- The amount of, and reactivity to some biogenic amines in the caecum of germfree versus conventional animals was determined (*Strandberg* et al., 1966).
- Caecal walls from four age-matched germfree and conventional rats, reared on the same autoclaved semi-synthetic diet (D7-diet), were investigated for the presence of some biogenic amines. Mean values, expressed a mcg/wet tissue in germfree and conventional rats respectively, were as follows: noradrenaline 0.56/0.98, l-adrenaline <10/<10, dopamine <10/<10, serotonin 5.8/6.5, acetylcholine 8.7/10.7, histamine 17.9/17.6. As is evident from these data, the concentrations of biogenic amines were virtually the same in both groups.

When investigating smooth muscle sensitivity towards these biogenic amines, strips from the same two groups of animals were prepared from the medium part of the caecum along the great curvature and movements were recorded isotonically on a smoked drum with a linear frontal writing level. Threshold dose was defined as the

smallest dose causing measurable concentration of the strip. Threshold doses, given as median value, mcg/ml bath fluid, for the four biogenic amines tested in groups of germfree and conventional animals were as follows: l-adrenaline 1.0/0.25, serotonin 0.75/0075, acetylcholine 1.5/0.005 and histamine >100/1.24. Thus, strips of caecal smooth muscles were 1-3 log less sensitive for biogenic amines than strips from their conventional counterparts.Also the types of contractions varied between the two groups of animals, most pronounced regarding serotonin. Strips from germfree animals subjected to serotonin showed an initially rapid, then slowly proceeding contraction. The strips from the conventional rats on the other hand reacted by rapid, rather twisting contractions.

From these experiments it was quite obvious that germfree rats had normal amounts of main biogenic amines in their colonic tissue. However the muscular sensitivity towards these amines was markedly reduced. As the focus at that time was on the microbes and mechanisms behind caecum enlargement, a second series of experiments was performed: Effect of conventionalization and mono-association with *Cl. difficile* of germfree rats on the caecum enlargement and reactivity to some biogenic amines (*Gustafsson* et al., 1970). In these experiments, age and gender matched young (at weaning) when included on the experiments and old(around 1 year old) germfree and conventional rats were used.They were all given the same autoclaved D7-diet. For Conventionalization, the faeces from 3-5 conventional rats were collected, suspended 1:19 in sterile saline, and each animal received aliquots of this suspension (1-2 ml) given both orally and rectally. After conventionalization, the animals were housed in the conventional animal room.

For mono-assosiation the strain *Cl. difficile* ATCC 0689 was grown anaerobically in Brain Liver Heart broth (Difco) for 3 days at 37°C, and aliquots of the broth were given to germfree rats as described above. These mono-associated rats were kept in the isolators during the experimental period. Female rats were only used for the comparison of smooth muscle activity in different organs while males were used in all other experiments. The most striking results were found in young animals. Conventionalization of germfree animals at weaning reduced caecum-size to conventional values within the observation period. Interestingly, the sensitivity towards the two biogenic amines acetylcholine and serotonin were similar in germfree and conventional rats at weaning, but were strikingly different 8 weeks later. The values found in conventionalized ex-germfree rats were comparable to those found in the conventional rats, whereas the values found in rats mono-associated with *Cl. difficile* were in-between the two groups. Interestingly, one of the rats in the *Cl. difficile* groups exhibited spontaneous contractions as those seen in conventional rats. In the old animals, the caecum-size was significantly reduced within the first week in both ex-germfree groups. The sensitivity towards the two biogenic amines was only modestly altered. Reactivity to biogenic amines in the other organs tested were similar, irrespectively of age and microbial status.

Before taking a glance in the historical mirror it has to be underlined that the focus for the research was on possible mechanisms behind the enlargement of caecum. *Cl. difficile* was chosen as a test organism because it was assumed that it belonged to an important part of the normal microbiota in young, healthy mammals. *Skelly* et al. (1962) found a full reduction of cae-

cum-size after mono-contamination with *Cl. difficile*, whereas *Wiseman* and (1965) found a transient reduction after mono-contamination of germfree rats with either *Cl. difficile* or *Salmonella typhimurium*. None of these investigators, however, studied alterations in sensitivity towards biogenic amines

When *Cl. difficile* in the 1970ties came up a causative microbe for antibiotic associated diarrhoea, the focus was changed. Out of the close to 6500 articles referred in Med-Line on *Cl. difficile*, only a very few are commenting on its motility properties, and here will only be mentioned a few. In 1998, Justus found that "*Cl. difficile* produces a heat labile substance or substances that altered the motility of the small intestine independent of the proteins responsible for *in vivo* tissue damage and cytotoxin assay positivity" (*Justus* et al., 1998). The substance(s) had a high molecular weight, were obtained from culture filtrates and "induced significantly more burst of action potentials (41.1/h) than all agents studied". In 2001, Huseby published studies on migrating myoelectric complexes (MMC), in the small intestine of germfree, conventional and ex-germfree rats mono-associated with *Clostridium tabificum*, *Lactobacillus acidophilus, Bifidobacterium bifidum, Echerichia coli* or *Micrococcus luteus* (*Huseby* et al., 2001). *Cl. tabificum* had the most pronounced effect, significantly reducing MMC-time in the mono-associated rats. *Cl. tabificum*, closely related to *Cl. difficile*, was chosen because it was known that it could influence upon some biogenic amines *in vitro*. Unfortunately the authors did not test *Cl. difficile* as a mono-contaminant. In 2000 it was shown in some *in vitro* studies that cytotoxin A was involved in "both direct excitation of enteric neurons and suppression of norepinephrine release from postganglionic sympathetic nerve fibres in the enteric nervous system" (*Xia* et al., 2001).

The results mentioned above clearly show that the intestinal microbiota do influence upon sensitivity towards biogenic amines and that *Cl. difficile*, by various mechanisms, most probably is involved in establishment and probably also maintenance of intestinal myelo-electric activity. The peculiar observation that both germfree as well as conventional rats at weaning had the same low sensitivity towards biogenic amines (*Gustafsson* et al., 1971) fits very well in a hypothesis that some basic functions are established at birth. If an intestinal microbiota is lacking, they might either be switched off or "re-started" when specific microbes are introduced (*Bry* et al., 1996).

CLOSTRIDIUM DIFFICILE AND INTESTINAL CELL KINETICS

In all mammals, the intestine is characterized with a very rapid turnover rate of enterocytes, supposed to be a major defence mechanism. In some comparative studies on germfree, conventional and mono-associated rats, we found that age, gender and microbial status influenced upon intestinal cell kinetics and morphology in an compartmentalized manner, and that most influences were related to the microbial status of the gut (*Banasaz* et al., 2000, 2002). In another series of experiments, groups of young, male germfree rats were mono-associated with either a toxin producing (Strain 70-685, gift from Prof. P. Bourlioux, Dept. of Microbiology, University Paris-Sud, Paris,

Table 2: Mitotic index (% of cells blocked in mitosis during 4 hours) in samples from jejunum, ileum, caecum and colon taken from rats mono-associated with a toxin producing and a non-toxin producing strain of *Clostridium difficile*

Day of mono-association	Toxigenic strain	Jejunum	Ileum	Caecum	Colon
0	yes	31	32	16	12
3	yes	39	37	25	20
7	yes	7	6	13	8
21	yes	33	32	18*	9
7	no	35	31	19	12

France) or a non-toxin producing (Strain CCUG 37785, Culture Collection, University of Gothenburg, Gothenburg, Sweden) strain of *Cl. difficile* for 3, 7 and 21 days, respectively. The mitotic activity was blocked, eight parts of the intestine were taken for microscopic examination, and aliquots from intestinal content were analysed for the presence of *Cl. difficile* and its toxins. All animals looked healthy and no diarrhoea was observed in any animal throughout the experimental period. *Cl. difficile* was found to be established in equal numbers in both groups. Toxins were found only in the group mono-associated with the toxin-producing strain, and highest values were recorded after 21 days (Table 2).

After 3 days of mono-associated, the mitotic index was found to be slightly elevated in all compartments in both groups. This is a common phenomenon observed when germfree animals are mono-associated with microbes including probiotic strains (*Banasaz* et al., 2002), and is assumed to reflect a general microbial triggering mechanism by substances present in both Gram-negative as well as Gram-positive microorganisms(*Oleya* et al., 2001). After 7 days, a dramatic reduction in mitotic activity was seen in the rats mono-associated with the toxin-producing strain. After 21 days, however, a normal mitotic activity was established again, irrespectively of presence of high amounts of toxins.

The clinical consequences of these findings can be outlined as follows: An increase in the number of toxin-producing *Cl. difficile* might cause a rapid and dramatic reduction in production of new enterocytes, i.e. a major defence mechanism in the intestine. However, for establishment of a disruption of the epithelial cell lining and development of diarrhoea, presence of other microorganisms might be needed. In some preliminary - and not yet published - experiments, we tried unsuccessfully to find such secondary troublemakers.

FUTURE ASPECTS

In future experiments, due considerations should be paid to combine the temporary increase in mitotic activity often found when some probiotic microbial strains are given together with the temporary decrease when toxin-producing strains are established. The results found in such models might be of prophylactic clinical importance. Additionally, characterization of possible secondary troublemakers should be intensified.

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BACTERIAL SPECIES AS PARTNERS AND PATHOGENS: SUMMARY OF THE SEMINAR AND THE DISCUSSIONS

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THE NOTION OF A BACTERIAL SPECIES

The 25th anniversary of the Old Herborn University Seminars (OHUS) provided an opportunity to delve into the concepts of bacterial species as friend and foe, as partners and pathogens. Many concepts have emerged and re-emerged during the past decade and have challenged our traditional notions of bacterial species. The definition of a bacterial species remains somewhat arbitrary despite advances in 16S rRNA gene sequencing, phylogenetic analysis, and bacterial genome sequencing. The cut-off value of 3% is still used frequently to distinguish bacterial species based on 16S rRNA gene sequencing data, but even this dividing line has been challenged for different genera and species. In this 2011 seminar, Pål Johnsen from the University of Tromsø discussed the nature and extent of sex and DNA transformation in bacterial species such as *Acinetobacter baylyi*, *Bacillus subtilis*, and *Escherichia coli*. Several bacterial species have developed sophisticated machinery for DNA uptake, and this facilitation of DNA transfer among microorganisms confounds phylogenetic analysis by permitting facile lateral exchange of genetic information (*Johnsen* et al., 2009). In addition to the contributions of sex to bacterial evolution and fitness, features that are potentially beneficial or deleterious to the host may be intermingled within one or several bacterial species. Individual species such as *E. coli, Clostridium difficile* and *Helicobacter pylori* may include a variety of strains that may be more or less virulent and perhaps beneficial to the host. Features such as quorum sensing may enhance intermicrobial communication in gut communities, and evolutionary pressures may be more relevant in the context of a single cell versus a community of microbes. In the single cell context, it may be difficult to reconcile the perceived importance of quorum sensing with the process of bacterial species evolution. The bacterial pangenome concept further challenges our ideas about bacterial species as partners and pathogens; a pangenome of any individual bacterial species may include pathogenicity and probiotic features present in different strains.

BACTERIAL CELLS AND COMMUNITIES

In the modern era of the microbiome and metagenomics, any consideration of bacterial species as pathogens or partners must include the concepts of microbial ecology. Leaving aside discussions regarding evolution, the idea of friendly versus pathogenic communities was presented by V. Young in

the context of Koch's postulates of infectious diseases. Mammalian intestinal bacterial communities consist of many diverse bacterial species, and these communities may protect the host against enteric infection. Freter's publication from 1955 (*Freter*, 1955) highlighted the adverse consequences and manifestations of a bacterial species as pathogen in the aftermath of communal destruction by antibiotics. Toxigenic *C. difficile* may ascend from status of colonizer to pathogen following the administration of specific antibiotics and corresponding effects on microbiome disruption. Recent evidence suggests that intestinal communities with relatively limited bacterial diversity may be predisposed to infections and chronic disease. The concept of disease-prone and pathogenic communities is emerging to accommodate ideas about pathobionts, symbionts, and the interplay between combinations of different bacterial species and the gut mucosa. Bacterial species may be partners if present in symbiont-dominated communities, and strains of the same species may be pathogens in disease-prone, pathobiont-dominated communities. The contextual information within bacterial communities may dictate to some extent whether bacterial species benefit the host or cause infection.

MICROBES AS PARTNERS

Beneficial microbes and probiotics are terms used to refer to friendly microbes and their role as partners in life. OHUS 25 spotlighted the potential roles of bacteria such as Segmented Filamentous Bacterium (SFB*), Lactobacillus reuteri* and *C. difficile* as friendly partners of mammalian hosts. Bacterial species may stimulate intestinal development and mucosal immunity, and provide signals that promote intestinal physiology and motility. Examples of these beneficial effects include the role of SFB in stimulating the differentiation of T lymphocytes and IgA-producing B lymphocytes (*Gaboriau-Routhiau* et al., 2009). *C. difficile* may regulate mitotic activity of mucosal cells, and microbe-derived biogenic amines may promote intestinal motility. An interesting connection is the description of the probiotic species *L. reuteri* and its role in promoting crypt cell proliferation and intestinal epithelial cell migration (*Preidis* et al., 2012). These examples reinforce the notion of bacterial species as partners with specific beneficial functions, depending on the strain of a bacterial species.

Our bacterial partners may be engaged in conversations with mammalian cells and organs. Interkingdom signalling provides opportunities to understand mutualism, commensalism and symbiosis in the host, and the expanding field of microbial endocrinology presents opportunities for understanding microbe-mammal crosstalk (*Lyte*, 2011). Growth of bacteria in the presence of neurochemicals such as catecholamines and dopamine may enhance the relative abilities of bacteria to colonize the GI tract and be our partners or pathogens causing enteric infections. Iron and iron siderophores may be an important consideration as catecholamines and other neurochemicals may scavenge iron from transferrin or ferritin. Intestinal bacteria may produce signals such as gamma-aminobutyric acid (GABA) and histamine that may have beneficial, although presently unknown, functions in the gastrointestinal tract (*Thomas* et al., 2012). GABA is a compelling tar-

get for studies of signalling in the enteric nervous system, and considerations of GABergic cells may include host-associated microbes. Conversely, intestinal bacteria such as *E. coli* may contain receptors for catecholamines and dopamine, and bacterial partner species may receive and alter their cellular behaviour based on the presence of host-derived signals.

MICROBES AS PATHOGENS

Bacterial species may cause infections by different mechanisms including invasion and toxin production. Bacterial species that may be friendly in one context may be pathogenic in a different context. An excellent example of the microbes discussed in OHUS 25 is *C. difficile*. This microbe may be functionally neutral and perhaps beneficial on the basis of regulation of cell proliferation in the intestinal mucosa. However, when bacterial communities are disrupted and depleted functionally by antibiotics, toxigenic strains of *C. difficile* may proliferate and become pathogens causing antibiotic-associated diarrhoea and colitis (*Chang* et al., 2008). Bacterial species as pathogens depend on intrinsic capabilities of disease-causing bacteria, in combination with the context of the microbiome. The bacterial species *C. difficile* serves as an excellent example of bacterial colonizer and infectious agent. Early in the first year of life, *C. difficile* is frequently present in infants and not associated with any disease phenotype. Is this species beneficial for early development and perhaps immune maturation? Toxigenic strains have the ability to cause disease in contrast to non-toxigenic strains of the same species, further emphasizing the importance of specific virulence genes and their regulation in bacterial genomes. Finally, the typical pattern of *C. difficile*-associated disease in the context of antibiotic treatment highlights the importance of the bacterial community in the outcome of bacterial species as partner or pathogen (*Chang* et al., 2008).

The pathogen *Helicobacter pylori* infects the stomach, and it tends to predominate in simpler gastric communities as discussed by Lars Engstrand in OHUS 25. Effectively, *H. pylori* diminishes the diversity of microbial communities in the stomach, resulting in a restricted community dominated by *H. pylori* and permissive for chronic disease caused by *H. pylori*. A pathogenic species such as *H. pylori* may be associated with different disease states such as peptic ulcer disease, atrophic gastritis and gastric cancer (*Giannakis* et al., 2008). These different disease states may depend on the combination of *H. pylori* strains with bacterial communities that differ in composition. For example, gastric bacterial communities with a greater abundance of *Prevotella* and *Streptococcus* species are present in the chronic condition of atrophic gastritis, and this disease state predisposes the human host to cancer. Our idea of the bacterial species as partner or pathogen is modified again by *H. pylori*, a species that is intimately associated with human history (*Linz* et al., 2007). This species may be an important example of "disappearing microbiota" proposed by Martin Blaser; these disappearing species (secondary to targeted antimicrobial therapy) may be associated with the onset of diseases due to their absence (*Blaser* and *Falkow*, 2009). For example, the elimination of *H. pylori* by antimicrobial agents may result in diminished predis-

position to gastric disease and increased predisposition to esophagitis. Bacterial species may be pathogenic at one site, and partners at a different anatomic site in the human gastrointestinal tract. Differences in composition and function of oesophageal and gastric microbiomes may contribute to different outcomes – bacterial species as partner or pathogen.

SUMMARY THOUGHTS

Our ideas regarding bacterial species are evolving rapidly in the era of the mammalian microbiome, metagenomics, and pangenomes. The individual bacterial species presented and discussed at OHUS 25 provide excellent examples of the challenges when we consider bacteria as our friend or foe. As we say in English, "it all depends." As our knowledge of the microbiome and microbial genomes expand, we hope to appreciate more fully the diversity of bacterial strains within a species and the contributions of bacterial neighbours in the microbiome. These dynamic interactions among microbes may determine the final outcome of bacterial species as partners or pathogens in an individual mammalian host.

LITERATURE

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