Old Herborn University Seminar Monograph 25:
Bacterial species as partners and pathogens.
Editors: Peter J. Heidt, Tore Midtvedt, Volker Rusch, and James Versalovic.
Old Herborn University Foundation, Herborn-Dill, Germany: 1-15 (2012).

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

PÅL JARLE JOHNSEN

Department of Pharmacy, Faculty of Health Sciences,
University of Tromsø, Norway

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.

LITERATURE

Andersson, D.I. and Hughes, D.: Muller's ratchet decreases fitness of a DNA-based microbe. Proc. Natl. Acad. Sci. USA 93, 906-907 (1996).

Avery, O.T., Macleod, C.M., and McCarty, M.: Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus Type III. J. Exp. Med. 79, 137-158 (1944).

Bacher, J.M., Waas, W.F., Metzgar, D., de Crecy-Lagard, V., and Schimmel, P.: Genetic code ambiguity confers a selective advantage on *Acinetobacter baylyi*. J. Bacteriol. 189, 6494-6496 (2007).

Baltrus, D.A., Guillemin, K., and Phillips, P.C.: Natural transformation increases the rate of adaptation in the human pathogen *Helicobacter pylori*. Evolution 62, 39-49 (2008).

Berka, R.M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X., Sloma, A., Widner, W., and Dubnau, D.: Microarray analysis of the *Bacillus subtilis* K-state: Genome-wide expression changes dependent on ComK. Mol. Microbiol. 43, 1331-1345 (2002).

Bernstein, H., Hopf, F.A., and Michod, R.E.: The molecular basis of the evolution of sex. Adv. Genet. 24, 323-370 (1987).

Bryan, A. and Swanson, M.S.: Oligonucleotides stimulate genomic alterations of *Legionella pneumophila*. Mol. Microbiol. 80, 231-247 (2011).

Charpentier, X., Kay, E., Schneider, D., and Shuman, H.A.: Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*. J. Bacteriol. 193, 1114-1121 (2011).

Cohan, F.M., Roberts, M.S., and King, E.C.: The potential for genetic exchange by transformation within a natural population of *Bacillus subtilis*. Evolution 45, 1393-1421 (1991).

Cooper, T.F.: Recombination speeds adaptation by reducing competition between beneficial mutations in populations of *Escherichia coli*. PLoS Biol 5, e225 (2007).

Cooper, T.F., Rozen, D.E., and Lenski, R.E.: Parallel changes in gene expression after 20,000 generations of evolution in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 100, 1072-1077 (2003).

Dorer, M.S., Fero, J., and Salama, N.R.: DNA damage triggers genetic exchange in *Helicobacter pylori*. PLoS Pathog 6, e1001026 (2010).

Dubnau, D.: DNA uptake in bacteria. Annu. Rev. Microbiol. 53, 217-244 (1999).

Elena, S.F. and Lenski, R.E.: Test of synergistic interactions among deleterious mutations in bacteria. Nature 390, 395-398 (1997).

Felsenstein, J.: The evolutionary advantage of recombination. Genetics 78, 737-756 (1974).

Gerrish, P.J. and Lenski, R.E.: The fate of competing beneficial mutations in an asexual population. Genetica 102-103, 127-144 (1998).

Griffith, F.: The significance of pneumococcal types. J. Hyg (Lond.) 27, 113-159 (1928).

Haijema, B.J., Hahn, J., Haynes, J., and Dubnau, D.: A ComGA-dependent checkpoint limits growth during the escape from competence. Mol. Microbiol. 40, 52-64 (2001).

Hoelzer, M.A. and Michod, R.E.: DNA repair and the evolution of transformation in *Bacillus subtilis*. III. Sex with damaged DNA. Genetics 128, 215-223 (1991).

Johnsborg, O. and Håvarstein, L.S.: Regulation of natural genetic transformation and acquisition of transforming DNA in *Streptococcus pneumoniae*. FEMS Microbiol. Rev. 33, 627-642 (2009).

Johnsen, P.J., Dubnau, D., and Levin, B.R.: Episodic selection and the maintenance of competence and natural transformation in *Bacillus subtilis*. Genetics 181, 1521-1533 (2009).

Johnsen, P.J., Townsend, J.P., Bohn, T., Simonsen, G.S., Sundsfjord, A., and Nielsen, K.M.: Factors affecting the reversal of antimicrobial-drug resistance. Lancet Infect. Dis. 9, 357-364 (2009).

Kondrashov, A.S.: Deleterious mutations and the evolution of sexual reproduction. Nature 336, 435-440 (1988).

Lenski, R.E.: A distinction between the origin and maintenance of sex. J. Evol. Biol 12, 1034-1035 (1999).

Levin, B.R.: The evolution of sex in bacteria. In: The evolution of sex: A critical review of current ideas. (Michod, R. and Levin, B.R., Eds.) Sinauer Associates, Sunderland (1987).

Levin, B.R. and Bergstrom, C.T.: Bacteria are different: Observations, interpretations, speculations, and opinions about the mechanisms of adaptive evolution in prokaryotes. Proc. Natl. Acad. Sci. USA 97, 6981-6985 (2000).

Levin, B.R. and Cornejo, O.E.: The population and evolutionary dynamics of homologous gene recombination in bacterial populations. PLoS Genet. 5, e1000601 (2009).

Libby, E. and Rainey, P.B.: Exclusion rules, bottlenecks and the evolution of stochastic phenotype switching. Proc. Biol. Sci. 3574-3583 (2011).

Maamar, H., Raj, A., and Dubnau, D.: Noise in gene expression determines cell fate in *Bacillus subtilis*. Science 317, 526-529 (2007).

MacFadyen, L.P., Chen, D., Vo, H.C., Liao, D., Sinotte, R., and Redfield, R.J.: Competence development by *Haemophilus influenzae* is regulated by the availability of nucleic acid precursors. Mol. Microbiol. 40, 700-707 (2001).

Michod, R.E., Wojciechowski, M.F., and Hoelzer, M.A.: DNA repair and the evolution of transformation in the bacterium *Bacillus subtilis*. Genetics 118, 31-39 (1988).

Mongold, J.A.: DNA repair and the evolution of transformation in *Haemophilus influenzae*. Genetics 132, 893-898 (1992).

Moran, N.A.: Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Natl. Acad. Sci. USA 93: 2873-2878 (1996).

Muller, H.J.: The relation of recombination to mutational advance. Mutat. Res. 1, 2-9 (1964).

Nester, E.W. and Stocker, B.A.: Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. J. Bacteriol. 86, 785-796 (1963).

Ochman, H., Lawrence, J.G., and Groisman, E.A.: Lateral gene transfer and the nature of bacterial innovation. Nature 405, 299-304 (2000).

Otto, S.P. and Lenormand, T.: Resolving the paradox of sex and recombination. Nat. Rev. Genet. 3, 252-261 (2002).

Palmen, R., Buijsman, P., and Hellingwerf, K.J.: Physiological regulation of competence induction for natural transformation in *Acinetobacter calcoaceticus*. Arch. Microbiol. 162, 344-351 (1994).

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., and Claverys, J.P.: Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science 313, 89-92 (2006).

Redfield, R.J.: Evolution of natural transformation: Testing the DNA repair hypothesis in *Bacillus subtilis* and *Haemophilus influenzae*. Genetics 133, 755-761 (1993).

Redfield, R.J.: Genes for breakfast: The have-your-cake-and-eat-it-too of bacterial transformation. J. Hered. 84, 400-404 (1993).

Redfield, R.J.: Do bacteria have sex? Nat. Rev. Genet. 2, 634-639 (2001).

Redfield, R.J., Schrag, M.R., and Dean, A.M.: The evolution of bacterial transformation: Sex with poor relations. Genetics 146, 27-38 (1997).

Rice, W.R. and Chippindale, A.K.: Sexual recombination and the power of natural selection. Science 294, 555-559 (2001).

Roberts, A.P. and Mullany, P.: Tn916-like genetic elements: A diverse group of modular mobile elements conferring antibiotic resistance. FEMS Microbiol. Rev. 35,

856-871 (2011).

Souza, V., Turner, P.E., and Lenski, R.E.: Long-term evolution of *Escherichia coli.* V. Effects of recombination with immigrant genotypes on the rate of bacterial evolution. J. Evol. Biol. 10, 743-769(1997).

Stewart, G.J. and Carlson, C.A.: The biology of natural transformation. Annu. Rev. Microbiol. 40, 211-235 (1986).

Thomas, C.M. and Nielsen, K.M.: Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3, 711-721 (2005).

van Sinderen, D., Luttinger, A., Kong, L., Dubnau, D., Venema, G., and Hamoen, L.: comK encodes the competence transcription factor, the key regulatory protein for competence development in *Bacillus subtilis*. Mol. Microbiol. 15, 455-462 (1995).

Wojciechowski, M.F., Hoelzer, M.A., and Michod, R.E.: DNA repair and the evolution of transformation in *Bacillus subtilis*. II. Role of inducible repair. Genetics 121, 411-422 (1989).

Wylie, C.S., Trout, A.D., Kessler, D.A., and Levine, H.: Optimal strategy for competence differentiation in bacteria. PLoS Genet. 6, e1001108 (2010).