Evolution of Host-Parasite-Parasite Interactions

*Caenorhabditis elegans* and its Microparasite *Bacillus thuringiensis*: Consequences of Experimental Evolution for Host-Parasite-Parasite Interactions

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Summary

The reciprocal evolutionary effects pathogens and their hosts have on each other are one of the most powerful selective forces in evolution, leading to adaptive phenotypic and genetic changes of both antagonists. In nature, bacterial infections often consist of more than one genotype. Since the host represents a limited resource, an interaction between the co-infecting genotypes is likely and potentially has fundamental effects on the interaction with the host. Nevertheless, most studies focus only on the interaction of parasite and host, ignoring within-host dynamics between co-infecting parasite genotypes. In my thesis, I focussed on both, the consequences of long-term host-parasite evolution for the interaction with a host and for parasite-parasite interactions. The first chapter is a comprehensive theoretical overview presenting the effects of multiple infections on virulence towards the host. It summarizes not only potential social interactions between the different co-infecting genotypes, but also discusses the relevance of their relatedness and resulting consequences for virulence. In the second chapter I present the results from a long-term evolution experiment using Caenorhabditis elegans as a multicellular host, singly infected with one of two different strains of its microparasite Bacillus thuringiensis. I found that both, coevolution with and adaptation to the host, led to rapid diversification of the clonal parasite populations into distinct clones. These clones showed strain specific phenotypic changes (i.e., killing rate and production of antagonistic substances) not only within, but also between replicate populations. In the third chapter one of these evolved clones was compared to its ancestral, non-evolved clone on the molecular level. By using next generation genome sequencing, I analysed the underlying genetic mechanisms that led to diversification within the clonal population presented in chapter two. In this study I demonstrated the importance of bacterial genomic plasticity for adaptation: the results revealed that changes were mainly caused by mobile genetic elements (MGEs), especially transposases and plasmids. Overall this thesis shows that the evolutionary selection pressure mediated by a multicellular host causes phenotypic diversification of the parasite. This change within and between parasite populations is reflected on both, the phenotypic and the genetic level.
General Introduction
General introduction

Host-parasite interactions

“Parasites are everywhere, affecting almost every aspect imaginable in the life of their hosts including physiology, behaviour, life histories and, by implication, the structure of entire ecosystems.”

(Paul Schmid-Hempel, 2011)

Paul Schmid-Hempel briefly sums up two major characteristics of parasitism: its omnipresence in nature, as there are almost no organisms being too small to have parasites (Hamilton 1990) and its huge impact on respective host organisms. Per definition, parasites (here the term is used including eukaryotic organisms, bacteria and viruses) cause harm to their hosts after infection leading to a reduction of host fitness (de Roode et al. 2008). Such a reduction in host fitness is called virulence and can be measured by any correlated trait, e.g., the decline in host reproduction, killing rate of the host or the time span between infection and host death. As parasites are usually smaller in size, mainly have a haploid genome, shorter generation times and larger population sizes, they have the potential to evolve faster than their host organisms (Mackinnon and Read 1999; Ebert 2000), indicating their importance as strong selective forces in evolution.

If a parasite successfully adapts to a host, the host should adapt its defence mechanisms in order to clear the infection from the body or to prevent further spread (Frank 1996). Subsequently the parasite has to evolve new strategies - this antagonistic nature of host-parasite interactions can result in continuous adaptation and counter-adaptation of host defence and parasite counter-defence (Van Valen 1973; Dawkins and Krebs 1979; Thompson 1994). Such reciprocal adaptive genetic changes in two antagonists, i.e., the particular host and parasite species, caused by the selective pressure one partner confers on the other, are referred to as host-parasite coevolution (Woolhouse et al. 2002). An often cited quote in this context is by the Red Queen in Lewis Carroll’s book Through the Looking Glass (1871): "Now, here, you see, it takes all the running you can do, to keep in the same place" to illustrate the force of continuous adaptation to maintain parity with other evolving species (Red Queen hypothesis, Van Valen 1973). However, although one of the antagonists in a coevolutionary
scenario will always be ahead, the other partner should always change accordingly to be on a par with the other one. That is why it has also been compared to an arms race, depicting the continuous pressure to adapt in pathogenicity and resistance (Dawkins and Krebs 1979). A persisting interaction over time can thus produce one of the highest selective pressures known in nature (Woolhouse et al. 2002) and be a key in structuring natural populations, driving coevolution (Thompson and Cunningham 2002; Harvell 2004).

**Experimental evolution**

In nature all interactions between parasites and their host organisms are influenced by a multitude of biotic and abiotic factors. These factors might change the level of parasite virulence as it has been shown for temperature (Konkel and Tilly 2000), host density (Lively et al. 1995; Bieger and Ebert 2009) or the host microbiome (Lopez-Medina et al. 2015) and thus they shape the outcome of the arms race between the host and its parasites. Under natural conditions, it is hard to disentangle phenotypic or genotypic changes caused by environmental factors from those resulting from the actual interaction. Furthermore, field studies are constrained by an incomplete characterization of ancestral populations, as phenotypes and genotypes within one population might show strong variation. These differences make it difficult to distinguish between ancestral variation and the variation caused by evolution (Fisher and Lang 2016). To circumvent this problem, experimental evolution in the laboratory became a powerful approach since the early 1970ies.

Experimental evolution provides the opportunity to test specific evolutionary hypotheses and their reproducibility by performing it simultaneously in more than one replicate population. It offers the possibility to vary individual parameters, something that is not feasible in natural populations. Additionally, most bacteria, fungi, and nematodes that are frequently used in these studies can be frozen in regular intervals during experimental evolution. Thereby they can be returned to at any time point in order to identify genetic changes, measure fitness traits, or to replay the evolution experiment (Fisher and Lang 2016). Furthermore it enables to compare the outcome at different time points during experimental evolution and to perform time-shift experiments, in which samples of host (or parasite) populations from different time points are tested in combination with samples of parasite (or host) populations from other particular moments in time (Gaba and Ebert 2009). Especially the analysis of genetic changes opened new perspectives in experimental evolution over the last decade, as the development of faster and much cheaper high throughput next-generation sequencing enables researchers to link changes in the phenotype with underlying mutations.
The design of an evolution experiment can range from simple to very complex setups that include many different variables. The most complex studies are coevolution experiments in which both interaction partners evolve, giving the opportunity to analyse dynamics and mechanisms of reciprocal adaptation. So called one-sided or unidirectional selection experiments are often run in parallel to these coevolution studies. There, one interaction partner adapts to its non-evolving antagonist, attempting to disentangle phenotypic or genotypic changes that result from adaptation from those that result from reciprocal coevolution (Rafaluk et al. 2015b). Both setups have extensively been used for experimental evolution in bacteria-phage systems, as they are easily cultivable in large quantities with very short generation times.

Although bacteria-phage models have been in focus for a long time, there is growing insight from multicellular, more complex host organisms. These include for example nematodes (see review by Teotonio et al. 2017), fruit flies (Kellermann et al. 2015), plants (Meaden and Koskella 2017) or beetles (Bérénos et al. 2011a; Rafaluk et al. 2015a). However, the number of studies is still comparatively low.

**Consequences of long-term host-parasite interactions**

It is obvious that continuous adaptation and counter-adaptation of host defence and parasite counter-defence can lead to a variety of phenotypic and genotypic changes in both antagonists. The most obvious expectation is that coevolution has tremendous consequences for virulence and resistance in a host-parasite system. In one of the first studies that experimentally proved this assumption, chemostats were used to evolve *Escherichia coli* and its respective coliphage for 22-48 days (Chao et al. 1977). The experimental setup contained two environments that differed in nutrient availability. Under limiting conditions phages evolved, which were able to infect bacterial clones being resistant to the wild type phage and vice versa bacterial clones that were resistant to these evolved phages. This shows that selection pressure mediated by resource limitations and reciprocal adaptation strongly affected virulence and resistance in bacteria and phages.

The coexistence of different resistant and non-resistant genotypes in the study of Chao and colleagues (1977) shows furthermore, that host-parasite interactions can lead to diversification within clonal populations. This is in line with several other bacteria-phage coevolution studies that revealed antagonistic coevolution as a driver of phenotypic and genetic diversification in both, bacteria and phage (Forde et al. 2008; Paterson et al. 2010;
Evidence from multicellular hosts is rather scarce (e.g., Schulte et al. 2010). Additionally, Buckling and Rainey (2002) found that coevolution with a phage drives diversification not only within, but also between different bacterial host populations, as each takes a subtly different coevolutionary trajectory.

In contrast, yet we understand far less about the dynamics of host–parasite interactions at the molecular level, although this is what is needed if we aim to understand the phenotypic effects completely. Again it is mainly bacteria–phage systems that have to date been used to analyse the genetics of host–pathogen coevolutionary change (e.g., Paterson et al. 2010; Kashiwagi and Yomo 2011; Scanlan et al. 2011; Meyer et al. 2012; Perry et al. 2015) and also here, studies on multicellular hosts are scarce (e.g., Schulte et al. 2010; Bérénos et al. 2011b; Kerstes et al. 2012; Masri et al. 2015).

**Model system**

For this thesis *Caenorhabditis elegans* and its microparasite *Bacillus thuringiensis* (Bt) were used as a host-parasite system to study consequences of experimental evolution. The nematode *C. elegans* is a major model species and was the first sequenced multicellular organism 20 years ago (The *C. elegans* Sequencing Consortium 1998). Nowadays a wide array of knowledge is available on the molecular, cellular, developmental and behavioural biology. However, information on the natural ecology is growing but mostly unknown. As strains of *C. elegans* have been propagated in the laboratory for thousands of generations (Nigon and Félix 2016), handling conditions have been widely standardized (Stiernagle 2006).

Bt is a bacterium that is mainly known for its high importance for agriculture. Proteinaceous crystals that are produced during sporulation are active against a range of insects as well as nematodes and have led Bt-based products become the best selling biological agents to date (Roh et al. 2007). Bt is a member of the *Bacillus cereus sensu lato* group, which comprises seven species (Priest et al. 2004) including the well-investigated, name-giving species *Bacillus cereus*, an opportunistic human pathogen and the agent of anthrax *Bacillus anthracis*.

**C. elegans**

Free-living *C. elegans* can be found in compost heaps and rich humus (Hodgkin and Doniach 1997), but more recently it has also been isolated out of decomposing plant material, such as fruits and thick herbaceous stems (Félix and Duveau 2012). In rich soil or compost, it is mostly found in a non-feeding stage (called ‘dauer’) (Barrière and Félix 2005, 2007), whereas rotting
substrates provide abundant microbial food for the nematodes so that newly hatched individuals pass through four larval stages (L1, L2, L3, L4) and reach the adult stage after three days (Frézal and Félix 2015). As their diet mainly consists of bacteria, they also constantly interact with various obligate and non-obligate parasites, including fungi, microsporidia, bacteria and viruses (e.g., Pradel et al. 2007; Troemel et al. 2008; Félix and Duveau 2012).

*C. elegans* has a very peculiar mode of reproduction: hermaphrodites (XX) can either reproduce by self-fertilizing, or by breeding with males (X0) but not with other hermaphrodites (Anderson et al. 2010). Males, occurring by non-disjunction of the X chromosomes at meiosis or in the progeny of male-hermaphrodite crosses, are usually present at a low frequency, depending on genotype and environment (Hodgkin and Doniach 1997). Worms potentially benefit from sexual reproduction and outcrossing, because it allows faster recombination of favourable alleles.

***Bacillus thuringiensis*** (Bt)

Bt is a gram-positive, aerobic bacterium, characterized by its rod-shaped morphology and the ubiquitous distribution in diverse environments including soil, rocks, aquatic environments, food and the gastrointestinal tracts of various insects and animals (Nicholson 2002) (see Figure 1)

![Figure 1](image)

**Figure 1:** Vegetative cells of Bt under a light microscope (A). After oral uptake by *C. elegans*, Bt spores can be seen in the midgut through the transparent worm (B, arrow).

This persistence in partially harsh environments is mainly based on the production of their robust endospores that are resistant even to extreme environmental conditions (Abriouel et al. 2011). These endospores are associated with Crystal proteins that are δ-endotoxins known as Cry-toxins, being produced during the sporulation phase of the growth cycle. The toxic, highly specific effect of Cry-toxins on target organisms has made Bt known worldwide as a
biological control agent (Lambert and Peferoen 1992). Target organisms include the insect orders Lepidoptera, Coleoptera, Hymenoptera and Diptera as well as nematodes (Bravo et al. 2007). The primary action of Cry-toxins is to lyse midgut epithelial cells in the host organism by forming pores in the apical microvilli membrane of the cells, resulting in cell death by osmotic shock (Aronson and Shai 2001; De Maagd et al. 2001; Bravo et al. 2010; Pardo-López et al. 2013). Toxin genes are mainly located on plasmids, of which cells can carry several. These plasmids can bear different toxin genes that can be exchanged between cells or even be lost (Schnepf et al. 1998; Aronson 2002).

Bt is furthermore considered to be a producer of antimicrobial substances, including peptide and lipopeptide antibiotics as well as bacteriocins (Stein 2005) (see Figure 2). This confers the cells with a double advantage in terms of their survival in different habitats or in co-infections of a host. They can not only outcompete other microorganisms using the same resources, they can also remain in a dauer stage by sporulation until conditions change again.

**Experimental evolution using *Bacillus thuringiensis* and *C. elegans***

Despite its importance as a model system, the use of *C. elegans* for experimental evolution did only slowly begin in the 1990s (e.g., evolution of aging by Johnson and Hutchinson 1993 or sexual selection by LaMunyon and Ward 1995, 1997, 1998, 1999). Nowadays a remarkably broad range of evolutionary questions has been addressed using *C. elegans* for experimental evolution, profiting from the intense research that has focussed on the genetics and functional biology of *C. elegans* (The *C. elegans* research community. 2005/2014). Its cryopreservability potentially also contributed a lot to its popularity, as it allows one to prevent genetic changes of ancestral stocks during laboratory maintenance, permits accurate evaluation of repeatability and gives the opportunity to analyse different time points of evolution (Denver et al. 2010; Estes et al. 2011). Although worms cannot match the short generation times or huge population sizes for example of bacteria-phage systems, long-term experiments are still feasible as it has been done by Katju et al. (2015) for over 400 generations. Conducted studies mainly focused on the maintenance of androdioecy (e.g., Stewart and Phillips 2002; Morran et

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**Figure 2**: Competition plate assay with different Bt strains showing inhibition by production of antagonistic substances. The strain in the agar is inhibited by the strain on the surface (spot), indicated by a clear zone around the spot (arrow). Variations in the extend of the spot indicate differences in inhibition intensity.
al. 2009), local adaptation (e.g., Schulte et al. 2011), sex determination (e.g., Chandler et al. 2012), cell development (e.g., Farhadifar et al. 2015) or population genetics (e.g., Estes and Lynch 2003; Denver et al. 2010).

In contrast, the crystal-toxin proteins of Bt are used extensively to control insect pests, thus this bacterium has always been of great agronomic interest but of rather little relevance for experimental evolution. Long term studies using Bt mainly focussed on the host side, elucidating the evolution of resistance to previously isolated Bt toxins (e.g., Tabashnik et al. 2005; Gassmann et al. 2012). Evolution studies using Bt as a pathogen are rare. However, a particularly interesting study by Garbutt et al. (2011) used the diamond back moth Plutella xylostella that causes massive damage in kale crops. Their experimental evolution approach did not only focus on consequences for the parasite, they also investigated the effects of multiple Bt infections on virulence. Multiple infections can lead to competition between different genotypes as host resources are limited. This competition can be mediated by the production of bacteriocins (see section Bacillus thuringiensis), a costly trait that potentially affects virulence. After their experiment was continued for four passages, one strain outcompeted the other completely, as selection led to an increase in between-strain competition. In contrast virulence and pathogen growth were reduced, potentially because the costs to produce virulence factors outweighed the costs of antagonism in an environment with a high risk for multiple infections (Garbutt et al. 2011).

Up to this time point there have been very few studies combining the use of C. elegans as a host organism and Bt as parasite in experimental evolution. The most comprehensive studies have been performed by Schulte et al. (2010, 2011, 2012, 2013) and Masri et al. (2015). They found remarkable genetic and phenotypic changes in the worms and the parasites after 48 (see Schulte et al. 2010) and 28 host generations (see Masri et al. 2015). For the parasite Schulte et al. (2010) observed an increase in virulence after coevolution, but a decrease in growth rate, indicative of adaptation costs. Worms vice versa increased in resistance, however, this also came along with adaptation costs, reflected in a reduced population size and a smaller body size. The experimental setup of Masri et al. (2015) contained beside other treatments a host-parasite coevolution and a parasite adaptation treatment, where the parasite adapted to a non-evolving host. In line with the other study, they found that coevolution led to the maintenance of virulence in Bt. But it also resulted in a loss of biofilm formation, a cooperative trait that includes public good secretion. This is potentially again an indicator for adaptation costs. The maintenance of virulence correlated with elevated copy numbers of the plasmid containing the nematicidal toxin genes. Parasite adaptation in contrast caused a decrease in
virulence but a higher infection load. Genotype composition also showed remarkable differences between these treatments, whereas one strain swept to fixation in all of the independent replicate populations under coevolution, the composition under conditions of host adaptation remained diverse. This indicates a higher competition between coinfecting strains and the selective advantage of the dominant strain under coevolutionary conditions. This study highlights on one hand the strong effects of reciprocal coevolution vs. parasite adaptation to a stable host on different life-history traits, on the other hand it shows the tremendous consequences for genotype diversity within a parasite population.

Interactions of co-infecting genotypes

As it has already been discussed, host-parasite interactions are shaped by a multitude of biotic and abiotic factors, however, one major biotic factor are potentially co-infecting genotypes, as most infections in nature consist of more than one parasite genotype (Read and Taylor 2001). Of course the different genotypes might not interact at all. But the simultaneous infection of the same host with more than one strain of the same or of different parasite species might also lead to within-host dynamics that potentially alter the interaction between parasites and host. For many infectious diseases, social interactions among parasites and virulence have been shown to be linked (e.g., in schistosomiasis see Gower and Webster 2005; malaria infections see Bell et al. 2006 or in the multidrug resistant pathogen *Pseudomonas aeruginosa* see Rumbaugh et al. 2009). Nevertheless, the nature of this relationship highly depends on the type of interactions involved and who the interaction partners are. Social interactions between parasites are characterized by the fitness consequences they cause for the interacting genotypes (Hamilton 1964a). These can either be positive (i.e., cooperation) or negative (i.e., competition) for both partners, be costly for the actor but beneficial for the recipient (i.e., altruism) or vice versa, be beneficial for the actor but not the recipient (i.e., selfishness) (Bose et al. 2016) (see Figure 3). Here, a brief overview of cooperation and competition is given, as they are discussed in depth in chapter 1.
Figure 3: Potential within-host interactions between co-infecting genotypes (grey and black circles). Fitness consequences can either be negative for both interaction partners if the genotypes are competing, or positive if they are cooperating. Competitive interactions can be divided into indirect competition by faster replication than others or direct through spiteful interactions e.g., the production of bacteriocins. Cooperation can be mediated by the production of public goods or alternatively by a more efficient, prudent host exploitation and not the fastest. It is also possible that co-infecting genotypes do not interact within the host.

Known mechanisms of microbial cooperation can so far be distinguished in two different forms of which one represents 'prudence' (Figure 3). Here the cooperating individuals aim on the most efficient host exploitation and not the fastest. Therefore they limit their resource use for the benefit of others, resulting in a higher fitness compared to single infections (West et al. 2002). The second strategy is a more direct cooperation in which the microbes donate costly public goods, generating benefits to any neighbouring cell being suitably equipped to profit. Public goods are for example products that enhance host exploitation (e.g., toxins or digestive enzymes), nutrient-scavenging molecules (e.g., siderophores) or products for biofilm production (e.g., adhesive polymers). As the production of these cooperative goods is costly and should thus not be active if it is not required, it is controlled by quorum sensing, a mechanism by which bacteria can coordinate density dependent behaviours (Diggle et al. 2007).

Both, prudent and public good cooperation can only remain stable if both partners have a fitness gain that exceeds that of single infections and if no cheaters occur. Cheaters reap the benefits of investment by their neighbours by not cooperating or cooperating less than their fair share without paying the costs themselves, representing a form of selfishness (West et al. 2007). Thus both cooperation strategies are expected to increase with relatedness as it reduces the probability for cheaters (Hamilton 1964a,b).

Whenever co-infecting genotypes are not cooperating, but competing with each other, the fitness consequences are in total negative for both interaction partners. Like cooperation, competition can also act in different ways (Figure 3). A direct, but very costly strategy for the actor and the sensitive recipient is spite (also referred to as 'interference competition'). One ubiquitous spiteful behaviour is the production of anticompetitor toxins named bacteriocins as
it has also been shown for Bt (see section "Bacillus thuringiensis") (Gardner et al. 2004). These are chemical compounds produced by all major bacterial lineages to suppress the growth of other bacterial strains, reducing total population growth. The inhibition range of bacteriocins is often (but not always) limited to members of the same species as the producer, suggesting a major role in competition with conspecifics (Riley and Wertz 2002; Riley et al. 2003). Since bacteriocins should target closely related bacteria but not clones, clones are protected from the toxic effects by a genetic linkage between the bacteriocin gene and an immunity gene that encodes a factor that deactivates the bacteriocin (Riley and Wertz 2002). Spite should thus be favoured under conditions of intermediate relatedness as not only clones but also far related bacteria are not affected by the respective bacteriocins (Buckling and Brockhurst 2008).

Another more indirect way of competition is mediated by the replication rate. Since the host contains only limited resources, parasite genotypes replicating faster can overgrow other genotypes and thus exploit the host faster but not necessarily optimally (Levin and Pimentel 1981; Bremermann and Pickering 1983; Levin and Bull 1994; Nowak and May 1994; Frank 1996; Chao et al. 2000).

Of course, next to cooperation, conflict and relatedness, within-host dynamics can be influenced by a variety of additional factors that might affect the outcome of parasite-host interactions. Multiple infections can generally include an endless number of co-infecting genotypes, leading to very complex interaction patterns including parallel competition and cooperation between different interaction partners. According to that, not only the number, but also the ratio of a certain genotypes (e.g., Karvonen et al. 2012) and the order of infection might shape within host interactions (Read and Taylor 2001; Hood 2003; Gower and Webster 2005; Ben-Ami et al. 2008). Furthermore, social behaviour can be influenced by the respective host-parasite system, as different host organisms can for example differ in their immune responses. Whereas an infection in one host leads to prevention of infection by another strain, the immune system of another host might facilitate its infection (Brown and Grenfell 2001; Cox 2001; Alizon and van Baalen 2008; Mideo 2009).

Specific aims of this study using the C. elegans - Bt model for experimental evolution

So far we have seen that long-lasting host-parasite interactions are driven by the selective pressure the antagonists exert on each other and can thus be a key structuring force in natural populations (Thompson and Cunningham 2002; Harvell 2004). It can affect host and parasite population dynamics (Thompson 1998), genetic diversity (Masri et al. 2015) and changes in virulence and resistance (Woolhouse et al. 2002). But it is also linked to increased phenotypic
and genetic diversification within populations and may thus represent an important driver of biodiversity (Brockhurst et al. 2004; Bérénos et al. 2011b). However, as discussed in the last section ("Interactions of co-infecting genotypes") these dynamics are not solely shaped by the interactions between the parasite and the host, co-infecting genotypes may also interact with each other. These within-host dynamics of different genotypes potentially strongly influence the host-parasite interplay, as they share the same resources. This illustrates the complexity of parasite-parasite-host interactions and the difficulties that occur during the analysis of relevant data.

The aim of the present thesis was to shed light onto these complex interactions of parasite-parasite-host interactions. By using an evolution approach that included not only a coevolution, but also a one-sided parasite adaptation treatment to a constant host, we were able to disentangle the effects of adaptation from those of the actual interaction. The experimental system consisting of C. elegans as a multicellular host and Bt as a microparasite offers new insights into more complex host-parasite systems with high agricultural relevance. With a focus on the parasite we wanted show that long-term interactions with a host lead to phenotypic and genotypic diversification of initially clonal populations. This illustrates the huge impact of host-parasite interactions as a structuring force of natural populations. Additionally it shows the rapid ability of the parasites' genome to adapt to changing conditions in order to enable transmission to new hosts. Furthermore this is the first study not only comparing the interaction between hosts and parasites after experimental evolution, but also focussing on antagonistic parasite-parasite interactions. The ability to outcompete co-infecting parasites has a strong influence on genotype diversity within populations. Getting insight into these dynamics could help to predict the outcome of multiple infections in future.

This thesis is divided into three chapters to answer the following questions:

**Chapter 1 - Virulence is not stable and optimal virulence depends on a variety of factors. How do co-infecting genotypes alter virulence compared to single infections? How can microorganisms interact in multiple infections? How do these interactions and potential other factors affect virulence?**
Chapter 2 - Host-parasite interactions can lead to increased phenotypic diversification within populations of both antagonists. In how far does experimental evolution with *C. elegans* lead to phenotypic diversification in clonal parasite populations of Bt? Does this diversification change the competitive behaviour of the parasites?

Chapter 3 - Phenotypic diversification is potentially associated with changes in the parasites' genome. What genetic consequences does adaptation to *C. elegans* have for a Bt clone?
Synopsis

Chapter 1

Multiple-genotype infections and their complex effect on virulence

It is known that infections in nature are often caused by more than one parasite genotype and several life threatening diseases are also likely to consist of infections by multiple parasite genotypes (Arnot 1998; Read and Taylor 2001). This review focuses on the consequences of multiple infections (parasites of the same species) on virulence. Therefore we first gave theoretical background on virulence and discussed why it should be optimal and how optimal virulence depends on the host-parasite system. In the next step we emphasized that virulence is not static and that genotypes of parasite and host, biotic and abiotic factors as well as multiple infections might have strong influence. The latter is then discussed in detail in the following section where we analysed why virulence should be different in multiple infections. We pointed out that reasons can be manifold, however, of importance are potentially interactions between co-infecting genotypes using the same host-resources. These interactions can involve cooperation or competition and have to date been found in different forms for several microorganisms. These different ways of competing or cooperating with other genotypes are presented in detail. In the last section we then gave insight into further parameters influencing virulence in multiple infections, e.g., the host immune system, relatedness or the number of co-infecting genotypes.

Chapter 2

Experimental evolution with a multicellular host causes diversification within and between microbial parasite populations – differences in emerging phenotypes of two different parasite strains

The force of continuous adaptation and counter-adaptation of host defence and parasite counter-defence in long-term host-parasite interactions can produce one of the highest selective pressures known in nature. Studies on bacteria-phage systems have shown that these interactions can lead to increased phenotypic and genetic diversification within populations of both antagonists. However, evidence from other host-parasite systems is scarce. Here, we performed an evolution experiment using C. elegans as a host infected with one of two strains
of Bt. We tested to what extent diversification of the clonal parasite population into distinct phenotypes is driven by selection. Therefore we compared results for parasites of both strains that evolved in the presence of a coevolving host with those that adapted to a constant host. Single clones were isolated out of different evolved replicate populations and tested for changes in virulence towards the worms and the production of antagonistic substances. We found that both treatments led to rapid diversification of the clonal parasite population into distinct clones that differed not only within but also between replicate populations. Although we found diversification for both strains, phenotypic changes were interestingly highly strain specific. Whereas in one strain the production of antagonistic substances was strongly influenced than host killing rate, in the other strain virulence drastically decreased and competitive ability was affected only little.

Chapter 3

Adaptation to a host drives activity of mobile genetic elements and plasmid loss in the microparasite *Bacillus thuringiensis*

In the previous chapter we have shown that host-parasite interactions can lead to phenotypic diversification. This diversification resulted in distinct clones deviating in various characteristics from their ancestral clone. In this chapter we used next generation sequencing (NGS) to disentangle the underlying changes in the genome of an evolved clone in comparison to its ancestral clone. Although bacterial genomes have been found to be stable (Patel 2016), they contain various flexible elements, allowing rapid adaptation to a host organism. This rapid adaptation is mainly mediated by genome rearrangements and horizontal gene transfers, plasmids, prophages and the activity of mobile genetic elements (MGEs). Here we found that adaptation to a constant host over ten host generations was sufficient to cause manifold changes in nearly all of these genetic elements. We show that several plasmids have been lost in the adapted clone, of which one can potentially be linked to the decrease in virulence in this clone. Furthermore, we show the massive activity of MGEs, namely transposases, distributed all over the chromosome. This chapter illustrates how bacterial genomes are capable of this quick adaptation and thus have the potential to change continuously in order to react to a changing host.
Chapter I

Multiple-genotype infections and their complex effect on virulence

Joy Bose, Michaela H. Kloesener & Rebecca D. Schulte (2016)

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Chapter I: Multiple-genotype infections and their complex effect on virulence

Abstract

Multiple infections are common. Although in recent years our understanding of multiple infections has increased significantly, it has also become clear that a diversity of aspects has to be considered to understand the interplay between co-infecting parasite genotypes of the same species and its implications for virulence and epidemiology, resulting in high complexity. Here, we review different interaction mechanisms described for multiple infections ranging from competition to cooperation. We also list factors influencing the interaction between co-infecting parasite genotypes and their influence on virulence. Finally, we emphasise the importance of between-host effects and their evolution for understanding multiple infections and their implications.

1. Introduction

Host–parasite coevolution is associated with high selection dynamics and has manifold consequences for hosts and parasites (defined in this article sensu lato, including viruses, prokaryotes and eukaryotes). However, parasites may not only interact with their coevolving host, but also with co-infecting parasites. In this article, we focus on co-infections by different parasite genotypes of the same species, although co-infections by different species are doubtlessly relevant as well. Actually, most infections in nature consist of multiple parasite genotypes (Read and Taylor 2001). In recent years, multiple-strain infections attracted considerable attention because of their prevalence in naturally infected hosts (Petney and Andrews 1998; Cox 2001; Balmer and Tanner 2011; Louhi et al. 2013). Advances in methods of genotyping revealed that several life threatening diseases are likely to consist of infections by multiple parasite genotypes (Arnot 1998; Read and Taylor 2001). For instance, a recent outbreak of dengue fever in India revealed that 20% of infections comprised multiple dengue serotypes (Bharaj et al. 2008). Similarly, in the case of human malaria, infected adults were simultaneously infected by more than five Plasmodium falciparum strains (Lord et al. 1999). Moreover, it has been well documented that irrespective of geographical regions, prevalence of parasite infection is positively correlated with mixed genotype frequencies (Louhi et al. 2013).
Most studies on multiple infections, both theoretical and empirical ones, have focused on the consequences of multiple infections on virulence. This is doubtlessly essential for predictions about consequences for host populations and epidemiology. While theoretical models initially assumed a specific type of competition between co-infecting parasite genotypes resulting in increased virulence, nowadays a diversity of theories and models exist. They reveal that manifold interaction mechanisms between co-infecting parasites are possible and that their consequences for epidemiologically relevant factors like virulence are rather complex.

Even though the importance of diverse infections has often been acknowledged, our understanding of virulence evolution in multiple infections is still limited (Read and Taylor 2001; Rigaud et al. 2010; Garbutt et al. 2011). Disentangling the virulence evolution among single and multiple parasite infections is essential for the management of disease severity, epidemiology and also in our dealing with emerging drug resistance (Read and Taylor 2001; Alizon et al. 2011).

This review focuses on infections by multiple parasite genotypes of the same species. First, we summarise theories about multiple infections with a focus on their effect on virulence. For this, we first introduce virulence and the concept of optimal virulence. We then explain why different interaction mechanisms between co-infecting strains may change virulence and list further parameters which can influence virulence. Finally, we suggest directions which future research on multiple infections should take. We point out that although virulence is an important measure and is without doubt crucial for epidemiology, it is impossible to understand the interaction between co-infecting parasites from simply measuring virulence.

2. Virulence

2.1. What is virulence?

Parasites per definition harm their hosts by decreasing their fitness (Read 1994). This harm is referred to as virulence, the prime factor describing parasites. Virulence is frequently measured as reduction in host reproduction, host death rate or time to death, but any other measure which correlates with the reduction in host fitness such as the decline in red blood cells in vertebrates infected with Plasmodium (Mackinnon and Read 1999b) is valid. Although parasites have to cause harm to access host resources, this harm can feed back negatively on the parasite. For example, due to a parasite attack a host population may drop in number which in return results in a drop of parasite number.
In most theoretical models, virulence is considered as increase in host mortality due to the infection. For some models, this assumption is essential (see Section 2.2). If empirical studies use a different measure for virulence, comparisons between empirical results and theoretical expectations may not be straightforward, since different measures for virulence can behave differently (Alizon and Michalakis 2015).

2.2. Why should virulence be optimal?

From an evolutionary perspective, the degree of virulence which maximises parasite fitness is optimal and should be selected. However, parasite virulence and fitness do not necessarily need to be linked; the association can be random (Fig. 1a) and virulence can be a non-adaptive trait (Schmid-Hempel 2011). This is, for example, the case in bacterial meningitis (Levin and Bull 1994). Bacteria like Streptococcus pneumoniae usually colonise the respiratory tract. Sometimes, however, they colonise the cerebrospinal fluid instead, causing severe damage to the central nervous system. This infection route is a dead-end; no transmission occurs from here. Thus, virulence is uncoupled from parasite fitness. Alternatively, parasites may depend on host survival for replication and transmission. In such a case, any increase in virulence may lead to an earlier host death which would in turn reduce parasite fitness (Fig. 1b). This scenario is also known as the avirulence theory (Ball 1943) since low virulence is selected. Here, parasitism might easily evolve to commensalism or mutualism if the parasite offers some advantage to the host. Conversely, parasites may depend on host death for replication and transmission. Then, virulence and parasite fitness are positively correlated (Fig. 1c) and high virulence is optimal. If parasites, however, depend on host survival for replication and on host death for transmission, an intermediate level of virulence can be optimal (Fig. 1d). If the host dies too early, parasite replication will be low. If it dies too late, transmission rate, i.e. the

![Figure 1](image_url)
number of transmitted parasite particles to a new host per duration of infection, will be low. In both extremes, parasite fitness is reduced and the virulence-maximising fitness is intermediate. This scenario is described in the trade-off hypothesis (Anderson and May 1982; Ewald 1983). It is perhaps the best-studied theory for the evolution of virulence using models (Bremermann and Pickering 1983; Massad 1987; van Baalen and Sabelis 1995b; Ebert and Bull 2003; Alizon et al. 2009; Alizon and Michalakis 2015) and empirical studies (Dwyer et al. 1990; Mackinnon and Read 2004; Jensen et al. 2006; Fraser et al. 2007; Doumayrou et al. 2013; Williams et al. 2014). However, the trade-off hypothesis makes specific assumptions (parasite reproduction correlates with virulence, it depends on host survival and parasites can only be transmitted by living hosts) which are not fulfilled in all parasite–host systems. While an optimal level of virulence is likely in many systems, the underlying reasons can differ to those proposed by the trade-off hypothesis.

2.3. Virulence is not static

Virulence can be influenced by a variety of factors. First of all, virulence is characterised by both the host and the parasite genotype (Read 1994). Depending on which parasite genotype encounters which host genotype, the virulence might differ dramatically. This has been shown by genotype-specific interactions between hosts and parasites (e.g., Webster and Woolhouse 1998; Schulenburg and Ewbank 2004; Salvaudon et al. 2007; Luijckx et al. 2011). Second, ecological factors are determinants for virulence. They can be differentiated into abiotic factors like temperature (Konkel and Tilly 2000) or availability of chemicals essential for virulence like iron and copper (Ding et al. 2014), and biological factors, such as host density (Lively et al. 1995; Bieger and Ebert 2009), host age (Izhar and Ben-Ami 2015), food availability (Jokela et al. 1999; Brown et al. 2000; Restif and Kaltz 2006), infection history (Ben-Ami et al. 2008), dispersal (Wild et al. 2009) or the host microbiome (Lopez-Medina et al. 2015). Finally, all these types of factors, i.e. genetic specificity and abiotic as well as biotic factors, are likely to interact and depend on the specific experimental conditions.
system used, which makes predictions for virulence in nature difficult. Virulence is also a fast-evolving factor, which might change in the course of an infection, and the optimal level of virulence might differ within and between hosts (Levin and Bull 1994). Finally, the optimal level of virulence can be influenced by multiple infections, which will be discussed in detail in Section 3.

3. Virulence in multiple infections

3.1. Why should virulence be different in multiple infections?

In multiple infections, parasites might not act differently compared to single infections. However, the host represents a limited resource and therefore competition between two or more co-infecting parasites is likely (see Section 3.2). Alternatively, parasites could cooperate for more efficient host exploitation (see Section 3.3). Conflict and cooperation are forms of social behaviour which are characterised by the net fitness consequences they entail for the actor and the recipient (Fig. 2) (Hamilton 1964a, 1970), including all costs and benefits. Since competition is costly for both the actor and the recipient, each parasite genotype’s fitness within single hosts is expected to be reduced in mixed compared to single infections. Cooperation, however, should per definition increase each parasite genotype’s fitness and thus total parasite fitness (the total number of parasites transmitted) since it offers the actors and recipients a fitness advantage. Note that cooperation can also entail costs, as long as the net fitness is increased.

Depending on the exact nature of the interaction, virulence can differ in mixed compared to single infections. This may have different reasons. First, parasites may alter their behaviour depending on the presence or absence of a
co-infecting parasite genotype. For example, parasites may adjust their replication rates or the conditional expression of costly factors (e.g. spiteful substances) important for the interaction with co-infecting parasites. Such conditional strategies are likely to be selected if both single and multiple infections occur frequently. Leggett et al. (2013) showed that viruses which evolved under conditions where they experienced single and co-infections, evolved a plastic virulence: they killed faster in co-infections than in single infections, resulting in high fitness under both conditions. Please note that plastic responses are not present per se, they need to evolve as consequence of alternating infection scenarios between single genotype and co-infections.

Second, parasites can evolve to be better adapted to co-infections. For example, faster replication or the general expression of spiteful substances may be selected. Theoretical models usually study how virulence can evolve in multiple compared to single infections. Both, conditional and evolved strategies of parasite genotypes in co-infections can influence virulence (Mideo 2009; Alizon 2013; Alizon et al. 2013) and therefore virulence should differ in multiple infections (Fig. 3).

While we summarise theoretical expectations on the evolution of virulence in Section 3.2, empirical studies for different interaction types between co-infecting parasites are listed in Table 1.

3.2. Conflict between co-infecting genotypes

3.2.1. Resource competition (indirect competition)

Since the host represents a limited resource, parasite genotypes replicating faster can overgrow other genotypes which exploit the host slower (Levin and Pimentel 1981; Bremermann and Pickering 1983; Levin and Bull 1994; Nowak and May 1994; van Baalen and Sabelis 1995b; Frank 1996; Chao et al. 2000). This idea is based on the trade-off hypothesis (see Section 2.2). If two parasite genotypes with different virulence strategies infect the same host, the more virulent one may have a fitness advantage: although these strains may have relatively lower fitness when compared to single infections, they grow faster than their competitor in double infections, finally causing host death. Within one host, the faster growing (and thus killing) strain has a higher fitness and wins the competition, resulting in a virulence level higher than optimal (Fig. 4a). Note that the total fitness of each parasite type depends on the frequency of single and multiple infections, and that for the evolution of virulence it is also important how fast and successfully parasites can infect new hosts (van Baalen and Sabelis 1995b). For example, a faster within-host replication rate may trade off against the re-infection
success. Although faster exploiting parasites will win within hosts, they have a selective disadvantage between hosts. In *Bacillus thuringiensis*, toxin plasmids are essential for host exploitation (Masri et al. 2015). However, plasmid loss may lead to an increased replication rate, which may be advantageous when competing with co-infecting strains (Garbutt et al. 2011). Thus, toxin-free strains can be faster replicators, but are unable to infect on their own. Also note that virulence should increase with decreasing relatedness because the competition and thus virulence should increase with decreasing relatedness (Bremermann and Pickering 1983; Nowak and May 1994; Frank 1996).

3.2.2. Spiteful competition
Parasites might also evolve strategies for direct but costly inhibition of their competitors. This behaviour is called spite or interference competition (Gardner and West 2004; Gardner et al. 2004). Virulence should be reduced for two reasons: First, the number of infecting parasites is reduced since they inhibit or kill each other. Second, spite is costly and should trade off against other traits like reproduction or the production of costly virulence factors (Fig. 4b). Please note that since spite is costly, it is likely that a conditional spite production evolves, i.e. that spiteful substances are only produced if competitors are present.
Spiteful behaviour is also expected to be influenced by relatedness (Buckling and Brockhurst 2008). If relatedness is high, i.e. the population is nearly clonal, spite should not be favoured since the costly spiteful substances would be inefficient or harm close relatives which would decrease indirect fitness. If relatedness is low, too many different competitors are present. Although spiteful substances may kill some competitors, they are unlikely to work against all. The fraction of competitors killed is too low for the costly production of spiteful substances to pay off. Therefore, spite should only be favoured at levels of intermediate relatedness. Since spite correlates with reduced virulence, virulence should thus be high at low and high relatedness whereas it should be low at intermediate relatedness. Only then are resources invested in spiteful behaviour instead of the production of virulence factors. An example of this spiteful behaviour is the production of bacteriocins (West et al. 2007), of which many pathogenic bacteria are capable. Bacteriocins are substances which generally inhibit or kill closely related strains (Riley and Wertz 2002).

3.3. Cooperation between co-infected genotypes

3.3.1. Prudent cooperation
This model is opposed to resource competition. Both models assume that exploitation, i.e. fast parasite replication, correlates with virulence. Prudent cooperation defines a strategy in which co-infecting genotypes exploit the host not as fast as possible, but as efficiently as possible, i.e. that both genotypes gain from exploiting the host together compared to single infections (West et al. 2002). Thus, an optimal virulence which maximises parasite fitness of both genotypes should be selected (see also Section 2.2). It is not always possible that both genotypes gain equally from the cooperation, but as long as both gain compared to single infections, cooperation should be selected. If one strain has a lower fitness compared to single infections, or if cheaters arise which do not cooperate, competition as described above (see Section 3.2.1) should evolve. The optimal level of virulence and the resulting

Figure 4: Schematic overview of different interaction mechanisms between co-infecting parasite genotypes by comparing single and double infections. Black houses represent living, grey houses, dead hosts. Ovals represent parasites; different genotypes are represented by different shadings. (a) Resource competition. If only single infections occur, the genotype with an optimal virulence has a higher fitness and should be selected. The genotype with a higher virulence has a relative fitness advantage when co-infecting with the optimal genotype. Thus, virulence increases in double compared to single infections. (b) In spiteful interactions, both the actor and the receiver have reduced fitness compared to single infections since they are either inhibited or have costs for spiteful behaviour. The actor has a relative fitness advantage in double infections. Since parasite growth is reduced, virulence decreases. (c) Both genotypes exploit the host more prudently and thus efficiently in double infections. The fitness of both genotypes is increased compared to the respective single infections. Virulence decreases or equals that of the least virulent strain. (d) If the public goods of two genotypes combined are more efficient, the parasites can shift their resource allocation to growth. Both strains have increased fitness compared to single infections, virulence is increased. (e) Cheaters of cooperation cannot exploit the host on their own. However, in a double infection they can replicate faster than the co-infecting strain since they do not pay the costs of cooperation. Virulence is reduced since fewer cells produce virulence factors.
parasite fitness can differ from the values of single infections (Fig. 4c). If, for example, a combination of virulence factors produced by different genotypes acts multiplicatively, virulence may be increased in co-infections. If a lower virulence level is optimal, variants with reduced production of virulence factors should be selected. Thus, host exploitation becomes less costly. Please note that a conditional strategy can also evolve, i.e. that the expression of virulence factors is adjusted depending on the presence or absence of a co-infecting genotype. The fitness of each genotype in multiple infections can further be increased if the parasite genotypes are related because their indirect fitness is increased by cooperation (Hamilton 1964a,b). Thus, with increasing relatedness between co-infecting parasite genotypes, competition decreases and cooperation (prudence) increases. As a consequence, virulence is low (Bremermann and Pickering 1983; but see Chao et al. 2000).

3.3.2. Cooperation by producing public goods

Pathogens can cooperate directly by producing costly public goods like toxins or nutrient-scavenging molecules (West et al. 2007). Different types of public goods, such as different toxins, may be more effective for host exploitation. Reduced public good production per individual may be selected, leaving energy for other traits like replication, resulting in increased virulence (Fig. 4d) (West and Buckling 2003). The exact mechanisms by which public good cooperation can enhance parasite fitness compared to single infections depend on the specific public good. Here, a plastic response may also be selected, i.e. public good production may depend on the presence or absence of co-infecting genotypes. As with prudence, public good cooperation is expected to increase with relatedness (Hamilton 1964a,b).

The drawback of public good cooperation is that cheaters can arise which use the public good but do not produce it, leaving the costs to the producers but also reducing their benefit (Chao et al. 2000; Brown et al. 2002; Schjørring and Koella 2003). If the public good is a virulence factor, cooperation by public good production can increase virulence, but it may be decreased if cheaters are present (Fig. 4e). Cheaters are more likely to arise if relatedness is low (Hamilton 1964a,b) and therefore virulence should increase with relatedness (Buckling and Brockhurst 2008).

The production of public goods, including many virulence factors, is often controlled by quorum sensing. Quorum sensing is a mechanism by which bacteria can coordinate behaviours which are only effective if a certain bacterial density is reached (Atkinson and Williams 2009). Every cell produces quorum sensing signalling molecules. Once a certain threshold of these molecules is reached, the bacteria show common behaviours, for example biofilm production,
sporulation or the production of virulence factors. Since quorum sensing molecules also represent public goods, their production is also prone to cheating. Thus, if relatedness is low, quorum sensing cheaters may arise which do not produce public goods, i.e. quorum sensing molecules. In this case the critical threshold for some common action such as the production of virulence factors will be reached later, when the bacterial population has reached a higher density. Thus, virulence will be retarded (Brown and Johnstone 2001).

3.4. Immune-mediated interaction

The host immune response may interfere with the interaction between parasite genotypes. It may do so by either preventing infection by one strain if another is present or by facilitating its infection (Brown and Grenfell 2001; Cox 2001; Alizon and van Baalen 2008; Mideo 2009). However, it remains unclear whether the parasites are able to manipulate host immune responses to their advantage or whether immune-mediated interaction is just a by-product of the host immune response. Not much is known about the influence of interactions with the immune system on virulence, but the order and dose of infections are potentially important determinants (Paul et al. 2004; de Roode et al. 2005a; Jäger and Schjørring 2006). Previously, studies only focussed on immune-mediated competition, but theoretically it is also possible that cooperation is prevented or facilitated by the immune system.

We would like to point out here that the interaction between co-infecting strains may not only be influenced by the host immune system but also by medical therapy (Gandon et al. 2001; Hansen and Day 2014) or by the use of pesticides. For example, in Plasmodium chaubadii, drug-sensitive strains can outcompete resistant strains in untreated hosts and drug treatment gives the drug-resistant strain a competitive advantage (e.g., Wargo et al. 2007).
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<th>Virulence relative to single infections</th>
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4. Further parameters influencing virulence in multiple infections

4.1. Relatedness

Relatedness between co-infecting genotypes is supposedly a key factor in the shaping of virulence. Highly related parasites may increase their indirect fitness by cooperating with relatives or individuals sharing the same key gene (West et al. 2007; Leggett et al. 2014). Therefore, high relatedness favours cooperation. Lower relatedness increases the risk of cheating or selects for antagonistic behaviour, so that competition is more likely. Relatedness can thus influence the interaction and virulence, depending on the exact interaction type (for details see Section 3). If parasites can sense whether the co-infecting genotype is related or not, they may adjust their behaviour accordingly, if a plastic, condition-dependent response has evolved.

Recently, it has been shown that the infection process can influence the genetic composition of a parasite community within hosts (van Leeuwen et al. 2015). Due to bottlenecks during the initialisation of the infection, relatedness within infections remains high, thus favouring within-host cooperation.

4.2. Genotype-specific interactions

Host–parasite interactions are commonly mediated by genotype-specific interactions (GxG interactions). Virulence thus depends on the specific host and parasite genotypes (see also Section 2.3). It has also been shown that virulence in multiple relative to single infections depends on the specific host and parasite genotypes (Bell et al. 2006; Vardozalik and Schall 2009). To date, three studies also revealed GxG interactions between co-infecting parasite genotypes (Seppälä et al. 2012; Bose and Schulte 2014; Louhi et al. 2015). Bose and Schulte (2014) showed that whether virulence is increased or decreased in multiple compared to single infections depends on the exact genotype combination of parasites. This indicates that the exact type of interaction between co-infecting genotypes, i.e. competition or cooperation, depends on the specific genotype combination.

4.3. Number of co-infecting genotypes

The number of co-infecting genotypes may influence virulence in three ways. First, relatedness decreases as the number of co-infecting parasite genotypes increases (Section 4.1). Second, a
complex interaction pattern might exist between the co-infecting strains. For example, imagine a triple infection in which two strains cooperate but at the same time compete against the third strain. It is hard to predict the consequences for virulence and it gets harder the more genotypes are involved. Third, every additional parasite genotype implies an additional challenge for the host immune system in terms of higher parasite diversity, which may influence virulence as well.

There is only a limited number of theoretical models available which allow more than two parasite genotypes (Nowak and May 1994; Lion 2013; Sofonea et al. 2015). Also, empirical data are scarce. Vardo-Zalik and Schall (2009) found that in Plasmodium infections in lizards there is no difference in traits concerning parasite reproduction between multiple and single infections. However, the diversity of the effects increases with increasing parasite clone number, indicating that the effects depend on the exact parasite genotype combinations within hosts.

4.4. Ratios of co-infecting strains

The degree of conflict and the efficiency of cooperation should depend not only on the number, but also on the ratios of co-infecting genotypes. In other words, if a competitor is rare, competition should be weaker than if it was frequent. If host exploitation is more efficient for a certain ratio of public goods produced by two different strains, a certain ratio of parasite genotypes may have a selective advantage. Not many studies have focussed on different ratios of co-infecting strains (Gower and Webster 2005). Karvonen et al. (2012) exposed fish to different ratios of co-infecting Diplostomum pseudospathaceum. They found that infection success is highest if strains occur in equal proportions. The most likely reason for this effect is that the host immune system is more challenged if two genotypes infect in equal proportions. However, it was not tested whether the ratios of the two genotypes change during infection. Interaction between parasite genotypes and also with the host immune system may give a parasite genotype a disproportional advantage over the other, which is likely to affect virulence. For example, an empirical study using two distinct clones of the rodent malaria parasite Plasmodium chabaudi in varying ratios found that an initially rare clone produced in multiple infections as many or more oocysts than in single infections and thus increased in frequency (Taylor et al. 1998). This indicates that ratios are crucial for the interaction between parasite genotypes and that they are likely to affect virulence.

4.5. Order of infection
Sequential infection is a critical determinant of intra-host competition (Read and Taylor 2001; Hood 2003; de Roode et al. 2005a; Gower and Webster 2005; Ben-Ami et al. 2008). Especially competitively weaker genotypes which are often less virulent may gain considerable advantage by establishing an infection prior to competitor strains. They may even become the dominant genotype (Ben-Ami et al. 2008). Thus, virulence may be changed if the order of infection differs.

4.6. Experimental system

Whether multiple infections differ from single infections, whether co-infecting genotypes compete or cooperate and how the interaction between co-infecting genotypes effects virulence depends crucially on the experimental system that is studied and how it is studied. In how far the model systems fit the assumptions of the theoretical models has to be taken into account. For example, it is essential whether parasites need to kill their host for replication and/or transmission or not, whether they are transmitted vertically or horizontally and whether sequential infection is possible or not. Thus, it is difficult to predict the consequences of multiple infections for specific host–parasite systems based on general models or on studies using different experimental systems.

4.7. Evolution of multiple infections

Interactions between co-infecting parasite genotypes are not static, they do evolve. They may evolve within hosts, but they may also evolve between hosts. Differential transmission can influence the composition of multiple infections and thus the interaction between co-infecting genotypes. Thus, the study of the interaction between co-infecting parasite genotypes should not only include within-host processes, but also the between-host level. This problem is described by the scale of competition (Frank 1998): Even if one genotype wins the competition within one host, it does not necessarily imply that it wins on the population level, since its fitness also depends on its population-wide frequency, the frequency of multiple infections and the frequency of re-infections (Anderson and May 1982; van Baalen and Sabelis 1995a; Day and Proulx 2004; Alizon and Lion 2011). The number of theoretical models also considering between-host processes and epidemiology is growing (Alizon and van Baalen 2008; Boldin and Diekmann 2008; Mideo et al. 2008; Choisy and de Roode 2010; Alizon et al. 2013; Sofonea et al. 2015). However, hardly any study considers how multiple infections evolve over time in the presence of an (coevolving) antagonist. We are only aware of three systems for
which evolution experiments were used to study multiple infections. First, Turner and Chao (1998, 1999) found that if *Pseudomonas* evolves under conditions which select for an increased multiplicity of infection by the RNA phage phi, virulence is reduced. The reduction in virulence is caused by the presence of defective cheater phages. Second, when comparing single and double *Bacillus thuringiensis* infections in a lepidopteran host, Garbutt et al. (2011) found that double infections select for reduced virulence and more spiteful behaviour. However, this study leaves many questions open. For example, they used a constant and not coevolving host population, but a coevolving host may also influence the interaction between co-infecting parasites. Furthermore, in double infections they combined a lepidopteran-pathogenic parasite strain with a coleopteran-, but not lepidopteran-pathogenic strain. Although this scenario is likely to occur in nature, it may not resemble the interaction between two pathogenic strains within a host, since only one strain is producing the virulence factors as public goods but both strains may use it (Raymond et al. 2007). Third, Ebert and Mangin (1997) allowed the host *Daphnia magna* to coevolve with its microsporidian parasite. They found indications that higher parasite diversity within a host may lead to the evolution of higher virulence. However, there was no control or test for diversity, as this result was only a side effect of another study. Thus, empirical evidence for the evolution of within-host interactions including between-host dynamics and its consequences on virulence evolution is missing.

5. Interference between mechanisms

Throughout the present review, we have pointed out in which ways parasites might interact within hosts and which factors might influence the interaction. We want to emphasize that parasites can interact in different ways at the same time, i.e. they may compete even though they also cooperate. *B. thuringiensis*, an insecticidal and nematicidal bacterium, on the one hand produces some of its virulence factors, the Cry-toxins, as a public good. On the other hand, *B. thuringiensis* is able to produce bacteriocins, spiteful substances to harm other strains (Garbutt et al. 2011). Such an interaction between different mechanisms is likely in most pathogenic bacteria, since many virulence factors are public goods (West et al. 2007) and many bacteria are capable of bacteriocin production (Riley and Wertz 2002). Another example would be *Pseudomonas* which produces siderophores, iron-scavenging molecules, as public goods (Lamont et al. 2002). Iron is an important co-factor for virulence in this system. *Pseudomonas* also produces bacteriocins (e.g., Bakkal et al. 2010). How these different mechanisms interact and shape virulence is mainly unknown.
Recently, a theoretical model was published which allows parasites to interact in different ways at the same time and which also considers between-host transmission. This model will help to understand complex interaction systems in multiple infections and their impact on virulence and epidemiology (Sofonea et al. 2015).

6. Conclusion

Multiple infections are common, and studies on multiple infections are becoming more frequent. Both, theoretical and empirical studies have shown that multiple infections affect virulence and have the potential to strongly influence epidemiology. Understanding multiple infections should thus be of concern for public health. However, the interaction between different parasite genotypes or even parasite species can be diverse, ranging from competition to cooperation, and it can be influenced by a variety of factors. Different mechanisms, i.e. conflict and cooperation, may apply to one and the same model system. To fully understand the interactions between co-infecting strains and ultimately their epidemiology, both conflict and cooperation need to be taken into account.

Most studies on virulence evolution in multiple infections only consider the optimal level of virulence within hosts, ignoring that between-host effects may strongly influence the evolution of virulence. It may also make a crucial difference whether parasites are confronted with a coevolving host population or a static, non-evolving host, as often used in experimental studies. Therefore, between-host mechanisms and evolution over several host generations under controlled conditions should be considered for a full understanding of multiple infections and their effect on virulence and epidemiology.

Virulence is a complex trait which is influenced by a diversity of ecological factors. The interaction between co-infected parasite genotypes is an additional and important variable. Virulence also depends on the way it is measured (Alizon and Michalakis 2015). Thus, virulence can be an indicator for the interaction between co-infecting genotypes, but in many cases may be misleading. It should therefore be used with care.

Acknowledgments

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Chapter II

Experimental evolution with a multicellular host causes diversification within and between microbial parasite populations—Differences in emerging phenotypes of two different parasite strains

Michaela H. Kloesener, Joy Bose & Rebecca D. Schulte (2017)

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Chapter II: Experimental evolution with a multicellular host causes diversification within and between microbial parasite populations—Differences in emerging phenotypes of two different parasite strains

Abstract

Host-parasite coevolution is predicted to have complex evolutionary consequences, potentially leading to the emergence of genetic and phenotypic diversity for both antagonists. However, little is known about variation in phenotypic responses to coevolution between different parasite strains exposed to the same experimental conditions. We infected Caenorhabditis elegans with one of two strains of Bacillus thuringiensis and either allowed the host and the parasite to experimentally coevolve (coevolution treatment) or allowed only the parasite to adapt to the host (one-sided parasite adaptation). By isolating single parasite clones from evolved populations, we found phenotypic diversification of the ancestral strain into distinct clones, which varied in virulence towards ancestral hosts and competitive ability against other parasite genotypes. Parasite phenotypes differed remarkably not only between the two strains, but also between and within different replicate populations, indicating diversification of the clonal population caused by selection. This study highlights that the evolutionary selection pressure mediated by a multicellular host causes phenotypic diversification, but not necessarily with the same phenotypic outcome for different parasite strains.
Introduction

The antagonistic nature of host–parasite interactions can lead to continuous adaptation and counter-adaptation of host defence and parasite counter-defence (Van Valen 1973; Dawkins and Krebs 1979; Thompson 1994) (here the term ‘parasite’ includes eukaryotic organisms, bacteria and viruses). Long term host-parasite interactions can thus produce one of the highest selective pressures known in nature (Woolhouse et al. 2002), potentially affecting factors that are important for many ecological and evolutionary processes. These factors include host and parasite population dynamics (Thompson 1998), genetic diversity (Schulte et al. 2010) as well as changes in virulence and resistance (Woolhouse et al. 2002).

Host-parasite coevolution is furthermore linked to increased phenotypic and genetic diversification within populations. Empirical evidence comes largely from bacteria-phage models. Marston et al. (2012) used chemostats to perform coevolution over ~170 generations between the marine cyanobacterium *Synechococcus* and a lytic virus. They found that in four replicate chemostats between 4 and 11 newly evolved *Synechococcus* with different viral resistance and between 4 and 13 newly evolved viral phenotypes with different host ranges emerged. Other studies on viral-bacterial coevolution support this data by revealing genetic (e.g. deletions in the phage tail fibre gene) and phenotypic changes (e.g. host colony shape) and thus within-population diversity of both antagonists (Lenski and Levin 1985; Mizoguchi et al. 2003; Forde et al. 2008; Paterson et al. 2010). However, evidence from multicellular host-parasite systems is comparably scarce (e.g., Bérénos et al. 2011a; Schulte et al. 2013).

Not all interactions between hosts and parasites lead to reciprocal adaptation and counter-adaptation in nature. Parasites are capable of rapid adaptation to their hosts (Ebert 1998; Mackinnon and Read 1999a); therefore coevolutionary studies are faced with the question of how much change is caused by adaptation and how much results from reciprocal coevolutionary interactions. However, under natural conditions the effects of coevolution or adaptation are challenging to test because of difficulties in disentangling the effects of environmental factors from the outcomes of the actual interaction between the antagonists. Since the early 1970s laboratory experimental evolution has become a powerful approach (Horne 1970; Cowlishaw and Mrsa 1975; Chao et al. 1977) with which to understand host-parasite evolution. It allows experimental manipulation to test specific assumptions about host-parasite coevolution. Furthermore it offers the opportunity to disentangle the effects of adaptation to the host from changes mediated by coevolution, by replacing the coevolving host with a stable non-changing host. Thenceforth bacteria-phage systems have been analysed.
particularly intensely (see Koskella and Brockhurst 2014), but there is growing insight from multicellular, more complex organisms (e.g., Webster et al. 2007; Schulte et al. 2010; Bérénos et al. 2011a; Rafaluk et al. 2015a).

In a scenario where only the parasite adapts and the host does not evolve, the parasite is expected to change in order to optimize adaptation. In that case the genotype which is best adapted to the host should spread in the population and diversity should be lower than in a coevolutionary scenario. Thus the level of diversification within and between populations of parasites should be lower when they adapt to hosts than when they coevolve with hosts. This assumption is supported by empirical studies revealing stronger evolutionary changes in parasites from the coevolution treatment, e.g. in genetic changes and number of mutations (Paterson et al. 2010; Kashiwagi and Yomo 2011), infectivity range (Poullain et al. 2008) or virulence (Masri et al. 2015) compared to a one-sided adaptation treatment. This demonstrates that antagonistic coevolution causes rapid and divergent evolution, at both the genomic and the phenotypic level, and is thus likely to be a major driver of evolutionary change within species.

Here we performed an evolution experiment using a genetically diverse population of the multicellular nematode *Caenorhabditis elegans* infected with one of two strains of the microparasite *Bacillus thuringiensis* (Bt). We asked to what extent selection drives diversification of the clonal parasite population into distinct phenotypes in the presence of a coevolving host, compared to one-sided adaptation to a constant host. By comparing evolved parasite clones we show differences in the level of phenotypic diversification into distinct clones with regard to virulence and the production of antagonistic substances. It is known that microorganisms produce a variety of inhibitory substances, e.g. bacteriocins, to harm competing genotypes (Riley and Wertz 2002). Bacteriocins usually display a high degree of target specificity against related bacteria (Tagg et al. 1976), whereas bacterial clones are protected from the toxic effects by genetic linkage between the bacteriocin gene and an immunity gene (Riley and Wertz 2002). Since the production of these inhibitory substances is metabolically costly and often involves cell lysis for their release, they should only be produced if competitors are present. Thus analysing the inhibition of ancestral clones by coevolved or adapted clones could be considered as an indirect measure for evolution or diversification, as it indicates genetic divergence from the ancestral clone. Furthermore we analysed whether the evolutionary trajectories are similar for two different parasite genotypes by generating clones derived from distinct evolution lines that were performed in parallel. Based on differences in their genetic background it is likely that different genotypes also vary in their
responses to antagonistic coevolution. Strikingly, despite the number of studies analysing consequences of coevolution, there are usually two infection patterns: either hosts are infected with one parasite strain (e.g., Morgan et al. 2005; Pal et al. 2007; Paterson et al. 2010; Morran et al. 2013) or with a mixture of different genotypes as mixed infections are abundant in natural populations (e.g., Bérénos et al. 2011b; Schulte et al. 2013; Masri et al. 2015). In contrast, our experimental design enables the comparison of single infections with two different strains that evolved under the same conditions. This allows us to draw conclusions concerning the extent to which results obtained for one strain can be generalized for other pathogenic strains. Furthermore the analysis of changes in virulence and the production of antagonistic substances reveals not only the consequences of reciprocal evolution for bacteria-host interactions but also for bacteria-bacteria interactions.

Material and methods

Experimental evolution

We analysed parasite material derived from a previous evolution experiment. For this we used a genetically diverse and outcrossed *C. elegans* host population to simulate natural conditions. The population was originally prepared by Henrique Teotónio by consecutive crosses among 16 natural isolates (Teotonio et al. 2012). Worms were infected with a single Bt clone of either strain MYBT18247 or MYBT18679 (hereafter abbreviated as 247 and 679, clones generated and kindly provided by the Schulenburg laboratory, originally derived from strains provided by the Agricultural Research Service Patent Culture Collection (United States Department of Agriculture, Peoria, IL)). These nematicidal strains differ in phenotype, genotype and crystal toxin production (Payne 1992; Payne et al. 1993; Schnepf et al. 2001; Schulte et al. 2010). 679 has been described as the more virulent strain (Schulte et al. 2010; Masri et al. 2015) and has a higher growth rate on agar plates (Schulte et al. 2010). It has two nematicidal Cry-toxins (Cry14Aa1 and Cry21Aa2) which are located on the same plasmid (BTI_23p) (Sheppard et al. 2016). For 247, one nematicidal Cry-toxin (Cry6B) has to date been described (Schulte et al. 2010). In general, Cry-toxins are activated in the insect gut where they bind to specific receptors and cause cell disruption and host death. The amino acid sequences of Cry-toxins have highly conserved as well as variable regions, which cause high host specificity (Palma et al. 2014). Toxins or complete plasmids on which toxins are located can be lost or exchanged between bacterial cells (Schulte et al. 2010; Masri et al. 2015; Sheppard et al. 2016).
The experiment had two selection regimes (figure S1): First, one-sided adaptation (hereafter named adaptation), where the parasite was allowed to adapt to a non-evolving *C. elegans* population taken from a stock culture at each transfer step. And second, host-parasite coevolution, where both antagonists were continuously allowed to coevolve with each other. These regimes were run with both parasite strains, resulting in four different treatments with 20 replicates per treatment. The experiment was continued for five weeks, corresponding to 10 host generations. All results shown are based on the analysis of parasite material from the 10th host generation. Detailed information on the experimental procedure is given in the supplement (file S2).

**Isolating single clones from evolved Bt populations (after 10 host generations)**

For the analysis of clonal differences within the replicate populations, we randomly selected five of the 20 replicates (hereafter named replicates A-E) from each treatment and from these we isolated 5 individual clones (figure S1). Thus, we analysed 100 evolved clones. To isolate clones, Bt populations were grown on nematode-growth medium plates (NGM, diameter 9cm) (Stiernagle 2006) for three days. Thereafter, five random colonies per population were picked with an inoculation loop and individually transferred to new plates. As Bt cells adhere strongly to each other, we repeated the inoculation process to new plates five times in order to obtain single clones.

**PCR for genomic background and presence or absence of Cry-toxin genes**

To control for cross-contamination each clone was tested via PCR using specific primers for 247 and 679. Additionally all clones were checked for the presence of certain Cry-toxin genes, i.e., Cry6B for 247 and Cry14Aa1 and Cry 21Aa2 for 679 (for further information see supplement S2). The loss of Cry14Aa1 and Cry21Aa2 corresponds to a loss of the whole BTI_23p plasmid (Sheppard et al. 2016).

**Inhibition assays**

Inhibition of the ancestral 247 and 679 clones by evolved and adapted clones was tested in a plate assay. Prior to the experiment (24h) liquid overnight cultures for each clone and the ancestral clones were prepared with 100 µl Bt in a concentration of $10^7$ spores/ml in 5ml LB and incubated in a rotary incubator (140 rpm) at room temperature.

100µl overnight culture of one ancestral clone was added to 5ml warm soft-NGM (contains 79.5% less agar-agar than NGM), carefully shaken and spread over a Petri dish (diameter 9 cm) containing a basis of a thin, bubble-free PF-layer (peptone free nematode growth medium).
All clones that were tested for inhibition were added as spots of 10µl overnight culture onto the solidified soft-NGM surface. Each plate contained a spot of the ancestral 247 and 679 clone, one randomized clone of each treatment and a negative control (LB medium). To avoid overlapping of the inhibition zones, all spots were separated by a distance of 23 mm and evenly spread over the plates.

Test plates were incubated for 5 days at 18°C and 70% humidity. A clearance zone around a spot on the agar surface indicated that the ancestral clone in the soft-NGM was inhibited by the clone in the spot. To determine the extent of inhibition as a proxy for competitive ability, plates were scanned and spot size and clearance zone size were measured with ImageJ (version 1.43u) and subtracted from each other. Every isolated clone was tested in three technical replicates against 247 and 679.

**Host killing assays**

As a proxy for virulence we measured killing rate of isolated clones towards ancestral C. elegans from the stock (see “Experimental evolution” for details). Worms were exposed to single clones in order to assess individual differences in virulence within and between replicates and treatments. Therefore 20 age synchronized hermaphroditic worms of the last larval stage (L4) were transferred into a “worm-ball” (diameter 5cm) (Sicard et al. 2007). Worm-balls contained a layer of PF-medium and were inoculated with 3.2x10^7 Bt particles and ad libitum *Escherichia coli* OP50 as a food source to prevent any effects caused by starvation. As a control we used the non-nematicidal Bt strain 407 Cry- (Lereclus et al. 1989) and as a reference for changes during evolution, ancestral clones were included. Prior to inoculation, Bt was grown for 6 days on nematode growth medium (NGM). Three days after exposure, the killing rate was measured as the number of dead worms divided by the sum of dead and surviving worms. The experiment was performed in three technical replicates for each isolated clone and 33 technical replicates for the ancestral clones and the non-pathogenic strain. Worm balls were stored at 18°C and at 70% humidity. When killing rate of ancestral worms was assessed for whole evolved populations, we followed the same protocol. Here, we tested biological replicates without technical replicates for virulence against ancestral worms (247: n\text{Ancestral}=16, n\text{Coevolution}=15, n\text{Adaptation}=14; 679: n\text{Ancestral}=18, n\text{Coevolution}=13, n\text{Adaptation}=13).

**Statistics**

All statistical analyses were performed using IBM SPSS Statistics Version 23. All data were transformed to z-scores (calculation: (measured value for evolved clone – mean\text{ancestral clone}) / standard deviation\text{ancestral clone}), which represent the n-fold standard deviation distance to the
mean of the ancestral clone. Thus, if the z-scores deviate from zero, evolved bacteria differ from the ancestral clone. Positive values indicate evolution of increased traits and negative values the evolution of decreased traits (i.e. inhibition or killing). All analyses were performed for each strain and measurement separately. We tested four independent hypotheses (figure S1):

A. Coevolution and adaptation treatments differ from their ancestor.

To test whether treatments generally differ from the ancestral clones, we tested whether they differ from zero with a 1-sample t-test. To avoid pseudoreplication, we calculated the means of the technical replicates and clones, resulting in one mean for each of the five replicate populations for each measure. Since two comparisons were calculated for each strain and measurement, we corrected for multiple testing using FDR-method (Benjamini and Hochberg 1995).

B. Coevolution and adaptation treatments differ from each other.

Our experimental design had a nested set-up: For each treatment (Coevolution, Adaptation), we chose 5 replicate populations for which we analysed 5 random clones in triplicate. To evaluate the impact of the different factor levels, we calculated GLM’s with “treatment” (Coevolution, Adaptation) as fixed factor and “replicate population(treatment)” (replicate populations A-E per treatment) and “clone(replicate population(treatment))” (clones 1-5 per replicate population and treatment) as random, nested factors.

C. Replicate populations diversify within treatments.

and

D. Clones diversify within replicate populations and treatments.

To be able to compare the variance caused by each factor level for each treatment (Coevolution and Adaptation) separately, we calculated GLM’s with “replicate population (replicate populations A-E)” and “clone(replicate population)” (clones 1-5 per replicate population) as random factors for each treatment separately.

Killing rate was also tested on a population level, i.e. the evolved population from which clones were isolated. To test whether the evolved populations differed from the ancestral clone, we tested whether the z-scores differ from zero using a 1-sample t-test. Similarly to the data on the clones, we corrected for multiple testing using FDR (Benjamini and Hochberg 1995).
Furthermore, we tested for differences between the treatments (Coevolution and Adaptation) using an independent samples t-test.
Results

Figure 1 A-D: Graphs show results of killing rate and inhibition intensity of ancestral 247 and 679 for both strains (247: A+B, 679: C+D) and treatments (Coevolution: A+C, Adaptation: B+D). Each box represents results of one replicate population (A-E, right side) each consisting of five clones (1-5, left side). A positive z-score indicates an increase in the focal trait compared to the ancestral clone (0, vertical line), a negative z-score a decrease. Data for 247 Coevolution, replicate B, clone 4 have been excluded due to a contamination.

Strain identity and presence of toxin genes:

All 679 clones were identified as 679. Only one clone (clone 4, replicate B) of the 247 coevolution treatment was contaminated and therefore excluded from further analyses. Furthermore all clones were tested for the presence or absence of Cry-toxin genes (Cry6Ba1 for 247, Cry21Aa2 and Cry14Aa1 for 679). Cry21Aa2 and Cry14Aa1 were missing in all 679 clones except replicate populations A, clone 3 of the coevolution treatment and C, clone 3 of the adaptation treatment. Cry6Ba1 was detected in all 247 clones except population A, clone 2 and population D, clone 5 of the coevolution treatment and population E, clone 5 of the adaptation treatment.
Figure 2: We tested killing rate of coevolved and adapted Bt populations towards ancestral C. elegans. Positive z-scores indicate an increase in killing rate compared to the ancestral clone (0, vertical line), negative z-scores a decrease.

A. Coevolution and adaptation treatments differ from their ancestor.

247

The ancestral 247 clone was tested against itself, but there was no case of self-inhibition. Compared to ancestral 247, coevolution led to a slightly non-significant increase of self-inhibition (1-sided t-test, t=-2.923, df=4, p=0.043; considered as trend after FDR-correction; table 1A). Both treatments lost inhibition against 679 (coevolution: 1-sided t-test, t=-4.060, df=4, p=0.015, significant after FDR-correction; adaptation: 1-sided t-test, t=-2.736, df=4, p=0.052, remains a trend after FDR-correction; table 1A). Inhibition of a strain that is not encountered during experimental evolution is predicted to be redundant. Neither coevolution nor adaptation changed killing rate (1-sided t-test, t≥-1.317, df≥4, p≥0.258; table 2A), also not if measured on the level of evolved populations (1-sided t-test, t≥-0.586, df≥13, p≥0.568; table 3A).
Table 1: Result overview for inhibition assays. Either the ancestral clone of 247 or 679 was inhibited. A significant deviation from 0 indicates a change in inhibition intensity of the evolved clones (averaged for populations) compared to inhibition by the ancestral clone (A). Part B shows whether the treatment (Coevolution and Adaptation) influenced inhibition intensity. Differences in inhibition between different replicate populations of the same treatment and between the five clones of the same replicate population are shown in part C and D. Significant results are highlighted in black, trends in italics. Signs in brackets indicate the direction of change in inhibition for significant differences and trends: (> ) increase, (< ) decrease, (< > ) varying between clones relative to the ancestral clone.

### Inhibition size

<table>
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<tr>
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<td>Adaptation</td>
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</tr>
<tr>
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<td>Coevolution</td>
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<table>
<thead>
<tr>
<th>B) Difference between Coevolution and Adaptation (GLM1)</th>
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</thead>
<tbody>
<tr>
<td>F</td>
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<tr>
<td>247</td>
</tr>
<tr>
<td>679</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C) Difference between replicate populations (GLM2)</th>
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<tr>
<td>247</td>
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<td></td>
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<tr>
<td>679</td>
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<tr>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>D) Difference between clones within replicate populations (GLM3)</th>
</tr>
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<tbody>
<tr>
<td>247</td>
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<td></td>
</tr>
<tr>
<td>679</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* cannot be calculated as inhibition and SD are 0. Adapted 679 clones never inhibit ancestral 679.

** FDR-corrected for multiple testing, significant differences considered as a trend afterwards.

1 GLM with treatment (Coevolution, Adaptation) as fixed factor and replicate population(treatment) (replicate populations A-E per treatment) and clone(replicate population(treatment)) (clones 1-5 per replicate population and treatment) as random factors.

2 GLM with replicate population(replicate populations A-E) and clone(replicate population) (clones 1-5 per replicate population) as random factors for each treatment (Coevolution, Adaptation) separately.
Table 2: Result overview for killing assays. A significant deviation from 0 indicates a change in killing rate of the single clones (averaged for populations) compared to the ancestral 247 or 679 clone (A). Effects on virulence by the two treatments (Coevolution or Adaptation) are shown in part B. Differences between the five replicate populations of one treatment and between the clones of one replicate population are shown in part C and D. Significant changes are highlighted in black, trends in italics. Signs in brackets indicate the direction of change in killing rate for significant differences and trends: (>) increase, (<) decrease, (<>) varying between clones relative to the ancestral clone.

<table>
<thead>
<tr>
<th>Clonal level</th>
<th>A) Deviation from 0 (1-sided t-test)</th>
<th>B) Difference between Coevolution and Adaptation (GLM)</th>
<th>C) Difference between replicate populations (GLM)</th>
<th>D) Difference between clones within replicate populations (GLM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>247</td>
<td>t: -1.317, df: 4, p: 0.258</td>
<td>F: 0.372, df: 1, 8, 0.559</td>
<td>Coevolution: 0.500, df: 4, 19, 0.736</td>
<td>Coevolution: 3.563, df: 19, 48, &lt;0.001 (&lt;&gt;)</td>
</tr>
<tr>
<td></td>
<td>Adaptation: -0.37, df: 4, p: 0.972</td>
<td></td>
<td>Adaptation: 1.470, df: 4, 20, 0.248</td>
<td>Adaptation: 5.971, df: 20, 50, &lt;0.001 (&lt;&gt;)</td>
</tr>
<tr>
<td>679</td>
<td>t: -8.034, df: 4, p: &lt;0.001 (&lt;)</td>
<td></td>
<td>Coevolution: 1.438, df: 4, 20, 0.258</td>
<td>Coevolution: 50.014, df: 20, 50, &lt;0.001 (&lt;&gt;)</td>
</tr>
<tr>
<td></td>
<td>Adaptation: -14.332, df: 4, p: &lt;0.001 (&lt;)</td>
<td></td>
<td>Adaptation: 0.938, df: 4, 20, 0.462</td>
<td>Adaptation: 9.203, df: 20, 50, &lt;0.001 (&lt;&gt;)</td>
</tr>
</tbody>
</table>

1 FDR-corrected for multiple testing, significant differences remained significant (black).
2 GLM with treatment (Coevolution, Adaptation) as fixed factor and replicate population(treatment) (replicate populations A-E per treatment) and clone(replicate population(treatment)) (clones 1-5 per replicate population and treatment) as random factors.
3 GLM with replicate population(replicate populations A-E) and clone(replicate population) (clones 1-5 per replicate population) as random factors for each treatment (Coevolution, Adaptation) separately.

The ancestral 679 clone was tested against itself, but there was no case of self-inhibition. In contrast to 247 clones, self-inhibition did not evolve in 679 clones as a consequence of evolution with the host (1-sided t-test, t≥1.0, df=4, p≥0.374; table 1A). In the adaptation treatment, inhibiting ability of 247, a feature which is predicted to be needless under the evolution conditions, was reduced (1-sided t-test, t=-6.964, df=4, p=0.002, significant after FDR-correction; table 1A). Both coevolution and adaptation caused a decrease in virulence (1-
sided t-test, t≤-8.034, df=4, p<0.001, significant after FDR-correction; table 2A). The loss of virulence was also present but weaker in the evolved populations (coevolution: 1-sided t-test, t=-1.867, df=12, p=0.086, remains a trend after FDR-correction; adaptation: 1-sided t-test, t=-2.725, df=12, p=0.018, remains significant after FDR-correction; table 3A).

**Table 3:** Result overview for killing assays on population level. A significant deviation from 0 indicates a change in killing rate of evolved populations compared to the ancestral clone of 247 or 679. Differences between populations of coevolved or adapted populations for each strain represent changes in virulence due to the treatment. Significant changes are highlighted in black, signs in brackets indicate the direction of change: (> increase, (< decrease in killing rate.

<table>
<thead>
<tr>
<th>Population level</th>
<th>Killing rate</th>
<th>A) Deviation from 0 (1-sided t-test)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>t</td>
<td>Df</td>
<td>p</td>
<td></td>
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<tr>
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<tr>
<td></td>
<td>247 Adaptation</td>
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<td>0.839</td>
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<tr>
<td></td>
<td>679 Coevolution</td>
<td>-1.867</td>
<td>12</td>
<td>0.086</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>679 Adaptation</td>
<td>-2.725</td>
<td>12</td>
<td>0.018* (&lt;)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* FDR-corrected for multiple testing, significant differences remained significant (black) or trends (black, italics).

**B. Coevolution and adaptation treatments differ from each other.**

**247**

Coevolution and adaptation treatments of 247 did not differ significantly, there was only a trend for a slightly higher inhibition of 247 in the coevolution treatment (GLM, F=3.562, df=1, 8.062, p=0.096; table 1B).

**679**

Coevolution and adaptation treatments of 679 did not differ significantly, but there was a trend for higher inhibition of 247 in the coevolution treatment (GLM, F=4.717, df=1, 8, p=0.062; table 1B).

**C. Differences between replicate populations within treatments.**

**247**

Coevolved 247 populations differed in their inhibition of 679 (GLM, F=8.337, df=4, 19, p<0.001; table 1C), which implies that evolution did not proceed similarly in the populations of the
coevolution treatment but in populations of the adaptation treatment. For all other measurements, there was no significant difference between replicate populations within treatments (GLM, F≤1.470, df=4, 19, p≥0.248, table 1C, 2C), suggesting that evolution took similar routes in replicate populations.

In neither treatment there was variation between replicate populations (GLM, F≤1.593, df=4, 20, p≥0.215, table 1C, 2C), indicative of parallel evolution.

**D. Differences between clones within replicate populations and treatments.**

There was significant variation in the inhibition of 247 between the clones within the populations of both 247 treatments (GLM, F≥7.780, df=19, 48, p<0.001; table 1D). If inhibition of 247 occurred, it was caused by only one of the five clones of a population. This was observed in four of five replicate populations of the coevolution treatment and in one population of the adaptation treatment. Single clones within replicate populations of 247 differed also significantly regarding their killing ability, both in the coevolution and in the adaptation treatment (GLM, F≥3.563, df=19, 48, p<0.001; table 2D). Some clones had a decreased, other clones had an increased killing ability compared to the ancestral clone.

There was no significant variation between clones within replicate populations concerning their inhibiting ability (GLM, F≤1.593, df=4,20, p≥0.215; table 1D). However, in both treatments, there was significant variation between the single clones regarding their killing ability (GLM, F≥9.203, df=20, 50, p<0.001; table 2D). Although the majority of clones decreased their killing ability, in both treatments one clone of all analysed clones had similar or stronger killing ability than the ancestral clone.

Detailed information on statistical tests is given in Table 1-3, results are shown in figures 1 and 2.
Discussion

Our results reveal that the effects of coevolution with, or one-sided adaptation to, a genetically diverse host lead to rapid diversification of the clonal parasite population into distinct clones that differ within and between replicate populations. Additionally, we detected strain specific responses to both treatments, resulting in different phenotypes in terms of competitive ability towards co-infecting genotypes and the maintenance of virulence.

Theory predicts that under conditions of high and low kinship the proportion of cells that are susceptible to secreted antagonistic substances like bacteriocins is low, resulting in a benefit that is less than the costs that the production entails and thus there should be no inhibition (Riley and Wertz 2002; Gardner et al. 2004). In our study, the highest kinship should be present in the ancestral clone populations. Consistent with theory, ancestral clones of both strains did not inhibit themselves. We assume that selection through coevolution or adaptation led to a diversification of the ancestral parasite clone into distinct clones and thus reduced kinship and favoured the secretion of antagonistic substances. This assumption is confirmed by some clones of the 247 coevolution treatment, which inhibited the ancestral clone of 247 (figure 1A). Conversely only one clone of the 247 adaptation treatment competed against the ancestral clone. Interestingly the direction of change was highly strain specific in our study, since there was no difference between the treatments (ancestral clone, coevolution and adaptation) for 679 clones when tested against their ancestral clone. Probably for this trait the extent of diversification was lower in 679.

Strikingly the competitive ability of both strains was reduced when confronted with the ancestral clone of the other strain. Due to the fact that the production of bacteriocins and other microbial defence systems as well as the maintenance of the respective genes and immunity genes is costly (Reeves 1972; Chao and Levin 1981; Kerr et al. 2002; Cascales et al. 2007), the reduction in competitive ability might be the result of the absence of the other strain during coevolution or adaptation with the host. Inglis et al. (2013) showed frequent and continuous loss of bacteriocin encoding plasmids in Escherichia coli evolving over 180 bacterial generations when no bacteriocin was encoded or when cells were resistant. Since bacteriocins in Bt are often located on plasmids (Abriouel et al. 2011) and are thus expected to be easily lost, superfluous bacteriocin genes may have been lost in favour of other traits like bacteriocin-production against present competitors or exploitation of hosts.

We further analysed differences in the production of antagonistic substances between treatments. As already mentioned, compared to adaptation to a stable host, coevolution to a
changing host favoured diversification in 247 as indicated by the evolution of self-inhibition. In the coevolution treatment four clones inhibited 247, whereas in the adaptation treatment only one clone inhibited its ancestral clone. Thus coevolution tends to reduce kinship and leads to competition for resources. Studies supporting our findings are scarce, since few studies have disentangled the selective consequences of coevolution compared to adaptation to hosts. To date studies on changes in competitive ability of the parasites are missing. The majority of existing empirical data comes from bacteria–phage interaction models (Poullain et al. 2008; Paterson et al. 2010; Kashiwagi and Yomo 2011; Scanlan et al. 2011), but also revealed the strongest changes in coevolution treatments. We are aware of only one multicellular host–microparasite study showing that there are distinct consequences for different life history traits in a host-parasite coevolution treatment compared to parasite adaptation (Masri et al. 2015). They found that coevolution of *B. thuringiensis* and *C. elegans* led to maintenance of virulence but to loss of biofilm formation, which includes public good secretion and is thus expected to be found under conditions of high relatedness (Griffin et al. 2004; West et al. 2007). In comparison, parasite adaptation caused a decrease in virulence but a higher infection load (Masri et al. 2015).

To reveal evolutionary patterns we tested whether replicate populations differ within treatments. If not, it could indicate parallel evolution. The latter was the case for 679 which showed no variation between replicate populations for both treatments and all measurements. For example, there was a drop in inhibition of 247 from the ancestral clone to the clones of the adaptation treatment. Yet there were no differences between replicate populations within this treatment, implying that all five replicate populations changed in the same direction. This supports the results of Paterson et al. (2010) where replicate phage populations grouped together genotypically among treatments. In contrast, the replicates of the 247 coevolution treatment differed significantly between each other in their inhibition of 679. These results are consistent with Buckling and Rainey (2002) who found differences in infectivity between replicate populations of a phage after coevolution with *Pseudomonas fluorescens*. Again, whether selection follows different trajectories between replicate populations seems to depend on the focal strain.

There is empirical evidence from a range of different model organisms showing that host-parasite coevolution also affects the clonal composition within host populations (including bacteria, beetles, water fleas and snails) (Buckling and Rainey 2002a; Haag and Ebert 2004; Decaestecker et al. 2007; Wolinska and Spaak 2009; Bérénos et al. 2011b; King et al. 2011; Paczesniak et al. 2014). However, if the host is constantly changing, better adapted parasite
genotypes can spread in the population, thus affecting not only host populations, but also the composition and diversity of parasite populations. Studies focusing on bacteria-phage interactions have shown remarkable parasite changes mediated by coevolution, e.g. within-population diversification into generalist and specialist infectivity range types (Poullain et al. 2008) or an increase in genetic diversity of the parasite population (Paterson et al. 2010; Scanlan et al. 2011; Marston et al. 2012). Here we see differences in inhibition between the clones derived from the same replicate population in two replicates of 247 coevolved clones inhibiting their ancestral clone. While the majority of clones in these replicates showed no visible inhibition, one clone (replicate population A, B) produced a comparably large amount of antagonistic substances. This indicates that the increase in inhibition of the ancestral 247 clone by 247 coevolution clones was mediated by the minority of clones. Considering these findings on the level of the whole population, the non-producing clones might benefit from the costly production of antagonistic substances by those high-producing clones or conversely they might be the targets of bacteriocin production by other clones. However, we did not test this but it suggests that population-based analyses of traits being affected by coevolution or adaptation might only reflect the responses of frequent clones and neglect the rare ones.

Since our experimental procedure was designed to select for parasite virulence, we expected the host killing rate to be maintained or increased in clones of the coevolution treatment compared to the one-sided adaptation. This assumption is supported by other studies showing that coevolution with a host particularly favours parasite virulence (e.g., Buckling and Rainey 2002a; Schulte et al. 2010; Hall et al. 2011; Masri et al. 2015; Rafaluk et al. 2015a). Here, 247 clones and populations generally maintained virulence as predicted, but evolution did not lead to differences compared to the ancestral clone (figure 1 A+B, figure 2). For 679 clones the opposite was actually the case: we observed a significant drop or even complete loss of virulence in nearly all clones in both treatments compared to the ancestral clone (figure 1 C+D). Rafaluk et al. (2015b) reviewed scenarios where selection for virulence led to a loss of virulence (e.g., Bérénos et al. 2009, 2011a). They emphasized the mode of parasite transmission during experimental selection as a major factor causing unexpected decreases in virulence. But interestingly, when we analysed the material at the whole population level, we only found a significant drop in virulence from the ancestral clone to the adapted populations. There was neither a complete loss of virulence nor a difference between coevolved populations and the other treatments (adapted populations or the ancestral clone; figure 2). Considering these results and our experimental design, we assume that the transmission mode was not the reason for the extreme loss of virulence in our experiment. We rather hypothesise that generating single clones by repeated culture in a rich medium (see materials and method
might have led to changes in the pathogenic characteristics of Bt, as described by Bizzarri et al. (2008). Pathogenicity in Bt is mainly caused by Cry-toxins. These are associated with Bt spores and once they are taken up by the host, they cause pore formation in the gut epithelium (Höfte and Whiteley 1989; Schnepf et al. 1998). Recently Sheppard et al. (2016) reported the instability of the plasmid BTI_23p that encodes Cry14Aa1 and Cry21Aa2 in 679. Virulence towards *C. elegans* in plasmid lacking clones decreased below 5% or was lost completely after ten weeks of serial passages on growth medium, demonstrating that BTI_23p is required for pathogenicity. Interestingly, the plasmid was not completely lost in the bacterial populations, but maintained at low frequencies. When we tested for the presence of these toxin genes in the isolated clones of both 679 treatments, we detected them in only two clones (replicate populations A, clone 3 and C, clone 3). Strikingly, the two clones in our study that still carried the Cry-toxins are those that maintained virulence, whereas all other clones decreased in virulence or even lost virulence completely, supporting the results of Sheppard et al. (2016). Thus serial culturing to generate single clones is likely the cause of plasmid loss and subsequently of virulence loss in 679 clones, but not in 247, highlighting again that evolution might take rather different routes in different strains.

We hypothesise that the analysis of further clones per population would reveal more highly virulent clones that maintained the plasmid, potentially indicating a division of labour between the clones of one population. The altruistic specialization of cell types resulting in increased production efficiency has been shown for other microorganisms including the *Bacillus* genus (Strassmann et al. 2000; Deng et al. 2015; van Gestel et al. 2015). Alternatively, virulence may change to maximize parasite replication and transmission (Anderson and May 1982; Alizon and Michalakis 2015; Bose et al. 2016). If virulence is too high, the host dies too early and parasite replication is low. If virulence is too low, the transmission rate to new hosts is low. In cases, parasite fitness is reduced and fitness is maximized at an intermediate virulence. Since we neither measured infection load nor transmission to new hosts, we cannot draw any conclusions about the relationship between virulence and parasite fitness. However, the high degree of parallel evolution between replicate populations of 679 and the fact that nearly all analysed evolved 679 clones lost virulence indicate that reduced virulence has a strong selective advantage linked to higher fitness. Further investigation is required to reveal whether a lower level of virulence is optimal in this system, whether cooperative behaviour exists or whether the observed differences are due to experimental handling.

Finally, we observed strong diversity between single clones and also between replicate populations, but not between treatments (Coevolution, Adaptation). We suggest that the
duration of evolution (10 host generations) may not have been sufficient to cause extensive
differences between the treatments. In contrast to our results, Masri et al. (2015) already
could observe differences between the treatments after 12 host generations. Their
experimental design differed to our study since they started experimental evolution with a
mixture of different clones while we started with single clones. This implies that there was no
 genetic diversity in our starting populations and that adaptation was based only on new
mutations arising in the clonal population, and not on clonal selection and recombination as it
can happen in a genetically diverse population. Perhaps the bacteria in our study simply took
longer time to adapt.

Conclusion

This is the first study comparing the effects of host-parasite coevolution and adaptation on
two clonal parasite strains. Furthermore, we assessed diversification not only based on
parasite virulence, but also based on parasite-parasite interactions by measuring
competitiveness between evolved and non-evolved parasite genotypes. Depending on the
parasite strain, selection affected different traits; while the production of parasite-antagonistic
substances was more strongly influenced than host killing rate in 247, virulence decreased
drastically in 679. These results indicate that selection leads to diversification, although the
resulting phenotypes can be highly strain specific therefore future studies should consider
comparing multiple parasite genotypes.
Author contributions

J.B. and R.D.S. designed the evolution experiment, M.H.K. and R.D.S. designed the experiments for the characterization of clones. J.B. and M.H.K. performed the evolution experiment. J.B. acquired the data for virulence on the population level. M.H.K. isolated clones and performed experiments (virulence, inhibition, and PCR) on the clones. M.H.K. and R.D.S. analyzed the data and wrote the manuscript.

Acknowledgements

We thank Vera Kühlkamp, Janine Terzenbach and Manuel Hanke-Uhe for experimental help. Thanks to the members of the Department of Behavioural Biology, University of Osnabrueck and to the members of the Bacillus-Invertebrate Cluster of the Priority Programme SPP1399 funded by the DFG for discussion of the data. Thanks to Sophie Armitage for reading and correcting our manuscript. Funded by projects SCHU2522/1-1 and SCHU2522/1-2 of the SPP1399 of the German Science Foundation.
Supplementary information

PCR to confirm genetic background of isolated clones and the presence of selected Cry-toxin genes

We used 15µl reaction volumes containing 7.0µl ddH₂O, 7.5µl peqGOLD PCR-Master-Mix S (Peqlab), 0.1µM of each primer (50µM) and 0.3µl vegetative cells of the respective clone in PBS. Detailed information about the cycling protocol, primers and product sizes are found in table S1 and S2.

**Table S1:** After the isolation of single clones out of evolved populations, each clone was tested for its genetic background via PCR to check for possible cross contaminations during the experiment or the isolation. Details are given in the table.

<table>
<thead>
<tr>
<th>Primer (5’→3’)</th>
<th>MYBT18247</th>
<th>MYBT18679</th>
</tr>
</thead>
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<tr>
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<td>GCTAACACACTCCTTTACT</td>
<td>TAATGCCTGGGGAATCATAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCAAGAACTATCGTGTCG</td>
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<td>'30 58°C</td>
</tr>
<tr>
<td>1' 72°C</td>
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<tr>
<td>Product size</td>
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**Table S2:** Each clone was further tested for the absence or presence of Cry-toxin genes. Details are given in the table.

<table>
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<tr>
<th>Primer (5’→3’)</th>
<th>Cry6Ba1 (247)</th>
<th>Cry14Aa1 (679)</th>
<th>Cry21Aa2 (679)</th>
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<tr>
<td>Forward</td>
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Experimental evolution - General protocol

Figure S1: Experimental design and focus of subsequent analyses.

Experimental evolution had two major evolution treatments, namely coevolution and one-sided adaptation treatment. In the coevolution treatment both antagonists, i.e. *Caenorhabditis elegans* hosts and *Bacillus thuringiensis* (Bt) parasites, evolved with each other. Offspring of worms that survived the exposure to the parasite were subsequently exposed to Bt populations obtained from dead worms. In the adaptation treatment, parasites adapted to a non-evolving host. Worms from the stock were exposed to Bt populations from dead worms. For both selection regimes worms were infected with a single clone of the Bt strain MYBT18247 or MYBT18679.
Prior to the start of the experiment, worms of an outcrossed, genetically diverse population (Teotonio et al. 2012) were adapted to the laboratory conditions, aliquoted and stored in the \(-80^\circ\text{C}\) freezer (note that they survive freezing, Stiernagle 2006). For the start of the experiment and throughout the experiment, worms were thawed and maintained on *Escherichia coli* OP50 inoculated Nematode Growth Medium (NGM) plates for few generations. The ancestral Bt clones were aliquoted and frozen after proliferation on NGM plates for 5 days. Prior to the exposure to nematodes, Bt was thawed, NGM plates (9cm diameter) inoculated with \(2.5 \times 10^6\) Bt particles and incubated for 5 days to ensure that the majority of cells transformed into spores as only spores are associated with toxins that are the main virulence factors. 2 days before the exposure, worms were age-synchronised and purified of bacteria by alkaline hypochlorite:NaOH treatment (“bleaching”) that kills all nematodes and bacterial cells with the exception of nematode eggs (Stiernagle 2006).

Experimental evolution was performed in “worm balls”, transparent plastic balls with a diameter of 5 cm, consisting of two halves, filled up with a layer of “Peptone free Nematode Growth medium” (PF) (Sicard et al. 2007). The worm balls force worms into constant contact with bacteria and subsequently minimize the escape behaviour of worms, which is relatively high in standard Petri dishes (Sicard et al. 2007). PF was used because it is unfavourable for bacterial proliferation, thus it ensures that spores cannot germinate into vegetative cells. *E. coli* OP50 was used as ad libitum food source to ensure sufficient nutrient supply for a whole week, thus they did not rely on Bt cells as food. Each worm-ball was inoculated with a total of \(3.5 \times 10^7\) Bt particles and ad libitum *E. coli* OP50 in PBS. After inoculation, worm balls dried completely in a sterile bench before adding worms. Host populations were always started with \(~150\) L4 larvae one day after inoculation by washing off bleached worm maintenance plates with M9-buffer and pipetting a volume containing 150 worms to the worm balls.

Both antagonists were transferred to new worm balls once per week (equivalent to two host generations). Killing Bt was selected by collecting up to 30 dead worms from each worm ball to a PCR tube filled with 100µl liquid PF (i.e. without agar agar) at day two and three of exposure. After 2 days of incubation, during which bacteria could use up the host resources and sporulate, the bacteria were pasteurized (10 minutes at 70°C) to prevent unwanted coevolution of food or possible contamination. Next, they were plated on NGM (5cm diameter), incubated for 5 days, washed off with PBS and used for worm ball inoculation as described above. Worms which were exposed for 5 days to Bt (coevolution treatment) or worm populations from the stock (adaptation) were bleached as described above. After two days, 150 L4 larvae were transferred to the inoculated balls via pipetting.
To avoid drift effects resulting from transferring only a part of the population, every second host generation 5% original genotypes of both antagonists were added. At each transfer (every second host generation), samples of both evolved antagonists were preserved. Evolved bacteria were frozen at -20°C and evolved worms at -80°C (Stiernagle 2006). The experiment was run at 18°C and 70% humidity.
Adaptation to a host drives activity of mobile genetic elements and plasmid loss in the microparasite *Bacillus thuringiensis*

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Chapter III: Adaptation to a host drives activity of mobile genetic elements and plasmid loss in the microparasite *Bacillus thuringiensis*

Abstract

Bacterial parasites can adapt remarkably fast to their host organisms due to dynamic changes of their genetic material, a phenomenon that is called genomic plasticity. This plasticity can be mediated by mobile genetic elements (MGEs) of which some might be activated in response to various host generated biotic and abiotic stresses. A previous study has shown that parasite adaptation to a non-evolving host (*C. elegans*) led to phenotypic diversification within clonal populations of the parasite (*Bacillus thuringiensis*). Here, we analysed the underlying genetic mechanisms that caused this change in individual clones leading to diversification using next generation genome sequencing. Additionally, we elucidated whether adaptation to a host acts as a stressor for the parasite genome and triggers the activity of MGEs. Indeed, our analysis revealed massive transposase activity within the chromosome of *Bacillus thuringiensis*. Furthermore, we found that adaptation led to the acquisition of an incomplete prophage and to the loss of plasmids in this clone while other plasmids were maintained. This highlights the importance of a flexible genome for the microparasite under selection pressure with a natural host.
Introduction

Bacterial pathogens have an enormous social and economic impact on their hosts. This includes emerging drug-resistant bacteria causing human diseases in hospitals all over the world, fast evolution of new, non-resistant pathogens and the devastation of crops resulting in huge agricultural losses (e.g. Mangano and Modiano 2014). Hence, understanding host-parasite interactions and the underlying causes and consequences of parasite adaptation to their host organisms have always been of major interest. Bacterial host-parasite systems present an excellent research model for the study of pathogenicity since they allow to track genetic adaptation over consecutive cycles of parasite and host generations. These antagonistic interactions of parasites and hosts are driven by the selective pressure mediated through the continuous conflict between the divergent interests of each partner (Combes 2000). Thus they are considered to be one of the most significant selection factors potentially leading to changes in both antagonists (Haldane 1949). Adaptation of parasites to their host organisms is often observed within short time periods, potentially due to the mainly haploid genome, shorter generation times and larger population size (Mackinnon and Read 1999a; Ebert 2000). Therefore parasites can evolve faster than their host organisms, as the absolute number of de novo beneficial mutations is expected to increase with replication rate and population size, leading to an advantage in the coevolutionary arms race (Ebert 2000).

The development of fast and affordable next generation sequencing (NGS) methods enabled whole genome as well as whole population sequencing experiments (Shendure and Ji 2008). It has revolutionized the identification of genomic determinants driving adaptive processes since it allows to track all genetic modifications in an evolving population within a reasonable time frame. In principle, it offers a basis for understanding parasite adaptation on a genomic level and for identifying potential genetic targets for intervention and disease control (e.g. see Jackson et al. 2011; Bayliss et al. 2017).

Overall bacterial genomes have been found to comprise a stable core-genome (Patel 2016), a vital characteristic for the survival of any organism (Vettone et al. 2014), as well as a dynamic accessory genome that enables fast adaptation to changing habitat conditions (Fouts et al. 2016). The accessory genomes contains various flexible elements, allowing genome plasticity on an evolutionary time scale (Darmon and Leach 2014). This plasticity is driven by genome rearrangements and horizontal gene transfers, mediated by plasmids, prophages and the activity of mobile genetic elements (MGEs) (Frost et al. 2005; Juhas et al. 2009; Donkor 2013; Darmon and Leach 2014). The latter are a large class of independent genomic entities that
inhabit a bacterial host genome and often carry genes that are beneficial for their host (Gordon et al. 2014). MGEs can represent a significant proportion of a species genome, with evolutionary rates in the order of $10^{-7}$ to $10^{-5}$ substitutions per site per year (Capy et al. 2000; Wilson et al. 2009; Morelli et al. 2010; Biek et al. 2015; Didelot et al. 2016; Patel 2016). The best-studied MGEs include transposable elements such as transposons and Insertion Sequences (IS), integrative conjugative elements (ICEs), as well as bacteriophages and conjugative plasmids (Frost et al. 2005; Siguier 2006; Bobay et al. 2014; McCarthy et al. 2014). Transposable elements are segments of DNA that are able to move and replicate within a genome, causing genomic rearrangements, mutations and duplications (Patel 2016).

Bacteriophages are viruses that infect bacteria, whereas they are observed more frequently in pathogenic than in non-pathogenic strains (Busby et al. 2013). They can transpose their DNA into a bacterial chromosome or plasmid as prophage or in self-circularized status. In former, they replicate passively in their host genomes (lysogenic cycle) or redirect the replicative system of the host to produce phage particles followed by cell lysis (lytic cycle) (Taylor 1963; Bukhari 1976; Kleckner 1981; Bobay et al. 2014; Darmon and Leach 2014). In contrast, a plasmid is extrachromosomal DNA that replicates autonomously. It can infect plasmid free cells by conjugation as a form of horizontal gene transfer (HGT) (Raymond and Bonsall 2013; Patel 2016). Plasmids are classically covalently closed, circular double-stranded DNA molecules, but linear double-stranded DNA plasmids have been found in an increasing number of species (Hinnebusch and Tilly 1993; Stewart et al. 2005).

Despite the obviously different characteristics of all MGEs, they have in common, that they exploit host-cell resources for their maintenances (Alberts et al. 1989). This introduces a genomic conflict to maximize MGE fitness at the expense of the replicative success of the host cell (Doolittle and Sapienza 1980; Orgel and Crick 1980; Burt and Trivers 2008; Werren 2011). The costs may differ depending on the respective element as they do not all pose the same life-or-death dilemma as bacteriophages do (Rankin et al. 2011). Nevertheless, the synthesis of additional proteins, the potential disruption of important functions by the integration into the chromosome or the creation of transmission structures, are all factors being costly for the cell (Rasched and Oberer 1986; Diaz Ricci and Hernández 2000; Lerat and Ochman 2004; Rankin et al. 2011).

The view of the MGEs as an primarily metabolic cost factor has been considerably challenged by a range of observations supporting a major role of MGEs in the structural and functional evolution of genes and genomes, indicating that these elements can also have beneficial effects (Kazazian 2004; Biémont and Vieira 2006). It has been shown that prophages can
protect the cell from subsequent infections by introducing defence mechanisms like restriction modification systems and CRISPR elements (Naser et al. 2017), generating a non-lysis signal, increase pathogenicity by introducing toxins (lysogenic conversion) or change microbial ecology, e.g., their growth rates (Edlin et al. 1977; reviewed in Bondy-Denomy and Davidson 2014; Erez et al. 2017). This implies that MGEs are neither just selfish nor carry only ‘junk’ DNA. They can also enhance the fitness and pathogenicity of pathogens as well as their defence against invading DNA and thus have important roles in the rapid evolution and adaptation of bacteria (Shintani 2017).

Transposable elements and prophages are known to usually be in a silent state but can be activated in response to different biotic and abiotic stresses (e.g., Frost et al. 2005). In our study we wanted to analyse whether adaptation to a host in a serial passage approach triggers the activity of MGEs in a bacterial pathogen. In a previous study we found that experimental adaptation causes rapid phenotypic diversification within clonal parasite populations (Kloesener et al. 2017). This diversification was potentially mediated through a high selection pressure that was not only caused by adaptation to a genetically diverse host population, but also by an intra-specific competition between co-infecting, potentially better adapted parasite genotypes.

Here we used an initially clonal population of *Bacillus thuringiensis* (Bt) that experimentally adapted to a non-evolving *Caenorhabditis elegans* (*C. elegans*) host population for ten host generations. Subsequently, a random parasite clone was isolated and compared to its ancestor on the phenotypic and genomic level. On the one hand, phenotypic analysis focussed on changes in host killing rate of the parasites and change in host fitness measured by host reproduction as proxies for virulence. On the other hand, we measured the production of antagonistic substances between different parasite genotypes as a proxy for deviation from the ancestral genotype and competitive ability. The genomic analyses focussed mainly on changes in MGEs and plasmid presence. Therefore we investigated the activity of MGEs by tracking serial transfers and recombination events by comparing the ancestral and the adapted clone.
Methods

One-sided host adaptation and isolation of single genotypes

We analysed parasite material derived from a previous evolution experiment (unpublished data). For this, we used a genetically diverse and outcrossed *C. elegans* host population to simulate natural conditions. The population was generated by Henrique Teotónio by consecutive crosses among 16 natural isolates (Teotonio et al. 2012).

Worms were infected with a single Bt clone of strain MYBT18679 (for details see Sheppard et al. 2016; Kloesener et al. 2017). In this experiment we used a one-sided adaptation approach (hereafter named adaptation), where the parasite was allowed to adapt to a non-evolving *C. elegans* population taken from a non-evolving stock culture at each transfer step (see Figure 1). The experiment was continued for five weeks, corresponding to 10 host generations. A detailed description of the experimental procedure can be found in the supplement.

Next, one single clone was chosen from an evolved population of the 10th generation. During a first screen this clone showed phenotypic changes in virulence and competitive ability compared to the ancestral clone. The DNA was extracted, the whole genome was sequenced and analysed to detect phenotypic and genetic changes compared to its ancestral clone.

Figure 1: Experimental design. Worms were infected with a clone of strain MYBT18679 (referred to as ‘ancestral clone’). After three days of exposure Bt was isolated out of dead worms to infect new worms from a stock culture. This was continued for 10 host generations in 20 technical replicates. From the resulting evolved populations, one random colony was picked from one random replicate. After repeated growth steps, the resulting single clone was used for phenotypic and genotypic characterization compared to the ancestral clone.
generate stock cultures. All results shown are based on the analysis of one parasitic clone from
the 10th host generation compared to its ancestor (Figure 1).

**Illumina sequencing of isolated clone and processing of raw data**

The genomic DNA was extracted from the singularized adapted clone using the MasterPure™
DNA Purification Kit (Epicentre, Madison, Wisconsin, US). Resulting DNA was quality checked
and subjected for whole genome sequencing done at the Institute of Clinical Molecular Biology
(IKMB) in Kiel, Germany. For this purpose TruSeq DNA libraries were prepared according to the
manufacturer’s protocols and sequenced on an Illumina HiSeq™ 2000 sequencing machine,
generating 100 bp paired-end strategy reads. Remaining sequencing adapters were removed
using cutadapt version 1.0 (Martin 2011) and the resulting reads were quality filtered using
FastQC (Babraham Bioinformatics, Babraham Institute, UK).

Spades 3.5.0 (Bankevich et al. 2012) was used to assemble processed reads. For scaffolding,
the resulting contigs were aligned to the reference chromosome of MYBT18679 using the
Mauve Genome Alignment software (Darling et al. 2004). Automatic annotation was carried
out with Prokka v1.11 (Seemann 2014) using Bt 407 (Sheppard et al. 2013) as species reference
and a comprehensive Bt-toxin database (including Cry, Cyt, Vip, Sip proteins as described
elsewhere (Hollensteiner et al. 2017)). The Prokka pipeline was applied using protein gene
calling by prodigal (Hyatt et al. 2010), rRNA gene prediction with RNAmmer 1.2 (Lagesen et al.
2007) and tRNA genes identification with Aragorn (Laslett and Canback 2004), respectively.
Additionally, signal peptides were predicted with SignalP 4.0 (Petersen et al. 2011) and non-
coding RNA species with an Infernal 1.1 search against the Rfam database (Eddy 2011).

Data analysis was done in cooperation with the Institute of Microbiology and Genetics at the
Department of Genomic and Applied Microbiology in Göttingen, Germany.

**Analysis of genetic changes in the adapted clone**

The annotated chromosome and plasmids of the evolved MYBT18679 clone were compared to
the MYBT679 reference by using the Artemis Comparison Tool software (ACT, release 13.0.0)
(Carver et al. 2005). Therefore, comparison files were generated online with WebACT (Abbott
et al. 2005). Additionally, mappings of the single read data using bowtie2 on the reference
genome were performed. Single nucleotide variations (SNVs) were calculated using the single
read data from the evolved clone and the MYBT18679 reference genome. To check for new
acquired genetic material, a draft assembly of the single clone NG data was performed using
Mira 4. A BLAST based comparison of the contigs from the draft assembly to the MYBT18679
Genome was used to identify recombination and gene acquisition events. Prophage sequences within the genome of the reference strain and the chromosome of the adapted clone were determined and annotated with the web-based phage search tool PHASTER (Arndt et al. 2016).

**Phenotypic changes**

Changes in phenotypic traits of the evolved clone with a special focus on virulence and competitive ability were analysed. Moreover, changes in virulence towards worms were measured by host killing rate and host reproduction three days post infection (see supplement). The competitive ability was measured against MYBT18247, a Bt strain that is known to be sensitive to antagonistic substances produced by MYBT18679 (Kloesener et al. 2017) and against the ancestral clone. Differences in virulence between the ancestral and the adapted clone were analysed using the Mann-Whitney U test. As the replicates of the competitive ability assay of both, the ancestral clone and the adapted clone, were tested on the same plate, we had a paired set-up and used the Wilcoxon test to analyse changes in inhibition against MYBT18247 and MYBT19679, respectively. Both analyses for virulence were performed in 19 technical replicates, competitive ability was measured in 25 technical replicates.
Results

**Genetic changes in the chromosome**

The result of the genome comparison of the ancestral Bt strain MYBT18679 and the adapted clone is visualized in Table 1. The chromosome of the ancestral MYBT18679 clone has a size of 5.475 Mbp. In contrast, the draft genome of the adapted clone comprises a chromosome of approximately 5.278 Mbp (Table 1). To identify the differences, a focus was set on genome rearrangements and horizontal gene transfers, mediated by prophages and the activity of MGEs, namely transposases. Furthermore, the analysis included the identification of smaller mutations, including deletions and insertions as well as SNPs.

Table 1: Overview of genome statistics for the MYBT18679 reference genome.

<table>
<thead>
<tr>
<th>Genome statistics of the MYBT18679 reference</th>
<th>status adapted clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>size [bp]</td>
<td>GC [%]</td>
</tr>
<tr>
<td>chromosome</td>
<td>5.475.881</td>
</tr>
<tr>
<td>plasmid 1</td>
<td>187.728</td>
</tr>
<tr>
<td>plasmid 2</td>
<td>135.158</td>
</tr>
<tr>
<td>plasmid 3</td>
<td>94.918</td>
</tr>
<tr>
<td>plasmid 4</td>
<td>39.783</td>
</tr>
<tr>
<td>plasmid 5</td>
<td>22.591</td>
</tr>
<tr>
<td>plasmid 6</td>
<td>15.831</td>
</tr>
<tr>
<td>plasmid 7</td>
<td>13.042</td>
</tr>
<tr>
<td>plasmid 8</td>
<td>11.276</td>
</tr>
<tr>
<td>plasmid 9</td>
<td>4.192</td>
</tr>
</tbody>
</table>

Information about Cry-toxins are implemented in column “Cry-toxins”. The column “status adapted clone” gives information about the presence or absence of the respective plasmid in the adapted MYBT18679 clone.

The genome of MYBT18679 comprises ten replicons, a single chromosome and nine plasmids including plasmid 5 and plasmid 6 that encode all identified Cry-toxins. Differences in prophage regions in the chromosome of the reference strain and the adapted clone were analysed using the phage search tool PHASTER (Arndt et al. 2016). In total, the analysis revealed eight prophage regions for the ancestral strain (46.3Kb - 9.4 Kb in size) and nine prophage regions for the adapted clone (57.8Kb - 9.4Kb in size, see Table 2). In both clones, four prophage regions were classified as intact, two as questionable and two in the reference and three in the adapted chromosome as incomplete, respectively.
Table 2: Comparison of prophage regions in the reference chromosome and in the adapted clone using PHASTER. Identified regions were compared based on PHASTER parameters including size, completeness, total number of proteins and sequence similarity. Regions of both stains that had high similarity in these parameters are depicted with grey background colour. Differences are displayed with white background colour. *comparison with all 8 prophage regions of the ancestral clone

<table>
<thead>
<tr>
<th>reference chromosome</th>
<th>adapted clone chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>region size completeness</td>
<td># total proteins region size completeness</td>
</tr>
<tr>
<td>1 46.3Kb intact 56 1</td>
<td>51.2Kb intact 47 64.3%</td>
</tr>
<tr>
<td>2 58.7Kb intact 59 2</td>
<td>57.8Kb intact 58 96.7%</td>
</tr>
<tr>
<td>3 26.4Kb intact 54 3</td>
<td>25.8Kb intact 53 96.7%</td>
</tr>
<tr>
<td>4 40.0Kb intact 55 4</td>
<td>38.9Kb intact 53 97.2%</td>
</tr>
<tr>
<td>5 40.0Kb questionable 51 5</td>
<td>38.8Kb questionable 49 96.6%</td>
</tr>
<tr>
<td>6 45.8Kb questionable 63 6</td>
<td>45.2Kb questionable 65 98.6%</td>
</tr>
<tr>
<td>7 15.5Kb incomplete 29 7 13.3Kb incomplete 25 85.6%</td>
<td>hypothetical protein and putative transposase</td>
</tr>
<tr>
<td>8 9.4Kb incomplete 16 8</td>
<td>9.4Kb incomplete 16 100%</td>
</tr>
<tr>
<td>9 42.6Kb incomplete 29 7</td>
<td>39.5Kb incomplete 26 75.6-77.7%*</td>
</tr>
</tbody>
</table>

Seven regions in the reference chromosome had a sequence similarity of >85% with corresponding regions in the adapted clone. The changes in the adapted clone leading to a deviation from 100% sequence similarity, were predominantly caused by changes in MGEs within these regions. This included mainly position changes of transposases and deletions of associated hypothetical proteins (for details see Table 2, regions 2-8). The comparison of region 1 revealed a sequence similarity of only 64.30%. This was caused by changes of nucleotide positions as well as basepair deletions within a base range of approximately 26,000 bp. This affected several phage related proteins in the adapted clone (see Table 2, region 1), while the other parts remained identical compared to the corresponding region in the MYBT18679 reference. The 32.6Kb big region 9 of the adapted clone is classified as an incomplete phage region. Notably, this region reveals sequence similarities of only 7.50-36.7% to all other detected prophage regions in the MYBT18679 reference chromosome. Thus it indicates that this region is not derived from any of these reference phage regions and is therefore newly acquired in the adapted clone.

Besides the comparison of prophage regions in the reference chromosome and in the adapted clone, we focussed on chromosomal changes caused by transposase activity. In total, in the reference chromosome 42 transposases were identified (Figure 2). These transposases can be subclassified into: (i) transposases for different insertion sequence elements, (ii) PD-(D/E)KK nuclease family transposases, and (iii) putative transposases in snaA-snaB intergenic regions. PD-(D/E)KK nuclease involved in nucleic acid metabolism (Mukha et al. 2013): DNA restriction (Roberts et al. 2003), bacteriophage λ recombination (Kovall and Matthews 1997), DNA damage repair (Ban and Yang 1998; Tsutakawa et al. 1999), Holliday junction resolution (Hadden et al. 2001; Nishino et al. 2001; Middleton et al. 2003) and RNA processing (Dias et al. 2009; Xiang et al. 2009; Yuan et al. 2009). In the adapted clone the majority of class (i) transposases is missing (including an associated hypothetical protein) or had deletions of up to...
400 bp which was also the case for class (ii) and (iii) transposases. In total, 34 of 42 transposases had changed in the adapted clone (see Figure 2). In total these genes explain 31.3 kbp of the chromosomal reduction of the adapted clone.

Figure 2: Location of transposases in the reference chromosome. In total, we found 42 transposases, their position is depicted by numbers. Black numbers represent transposases that have been affected in the adapted clone. Grey numbers represent transposases that did not change their positions within the chromosome.

Genetic changes on plasmids

Besides the chromosome, the genome of the ancestral clone contains nine plasmids ranging in size from 4.2 kb – 187.7 Kb (see Table 1). As seen in the previous section, the reference genome harbours several different located prophages in the chromosome. Moreover, PHASTER also predicted seven prophage regions on plasmids 1, 2 and 4 (see Table 3). For the other five plasmids no phage region was detected.

Table 3: Comparison of detected prophage regions on plasmids. Investigations were done for MYBT18679 and the adapted clone.

<table>
<thead>
<tr>
<th>reference plasmids</th>
<th>similiarity of matching contigs in adapted clone [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasmid size [Kb]</td>
<td>plasmid size [Kb] region size [Kb] # total proteins</td>
</tr>
<tr>
<td>1</td>
<td>187.7</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>135.2</td>
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<tr>
<td></td>
<td></td>
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</table>

On plasmid 1, five prophage-like regions have been identified. PHASTER characterized one region as complete, three regions as incomplete and a last one as questionable. The positions of prophage regions 2 and 3 as well as regions 4 and 5 are overlapping (Table 3). Considering the reliability of the prediction tool PHASTER which is dependent on known references, this might as well indicate that the overlapping regions represent so far unknown single, complete prophages. On plasmid 2, an incomplete prophage region was predicted and on plasmid 4, two regions were found of which one was incomplete and one was questionable (Table 3). None of these prophage regions have been lost in the adapted clone or underwent massive changes as the similarity to the ancestral plasmids ranged between 95-100 % (see Table 3). The
comparison of the adapted clone to the reference has shown that four of nine plasmids have been lost after adaptation to the host, including plasmid 5, the plasmid encoding the nematicidal toxins. The five remaining plasmids involve plasmids 1, 2, 3, 4 and 6 (Table 1).

**Phenotypic changes**

The analyses of phenotypic traits of the adapted clone included two different measurements for virulence, namely host killing and parasite induced change in host reproduction three days post infection. Furthermore it included two measurements for competitive ability, inhibition of another nematicidal Bt strain, namely MYBT18247, and of the ancestral MYBT18679 clone (Figure 3).

The killing ability was significantly reduced in the adapted clone compared to the ancestor (Mann-Whitney U Test, n=19, U=93.5, p=0.008). Worms that were infected with the adapted clone produced significantly more offspring than worms that were infected with the ancestral clone (Mann-Whitney U Test, n=19, U=18.0, p<0.001). In contrast, there was no change in competitive abilities, neither against MYBT18247 nor against the ancestral MYBT18679 clone (Figure 3).
Figure 3: Phenotypic results for the adapted MYBT18679 clone in comparison to the ancestral clone (horizontal line). Shown are results for killing rate of worms from the stock, reproduction of worms three days post infection, inhibition of another pathogenic strain (MYBT18247) and inhibition of the ancestral MYBT18679 clone. For this graph we transformed the data into z-scores (calculation: (measured value for evolved clone – mean value of the ancestral clone)/standard deviation of the ancestral clone), which represent the n-fold standard deviation distance to the mean of the ancestral clone. For the statistical analysis we used original values. A positive z-score indicates an increase in the focal trait compared to the ancestral clone (0, horizontal line), a negative z-score a decrease. Circles represent outliers.
**Discussion**

This study shows that adaptation to a genetically diverse nematode host population over ten host generations was sufficient to cause several genetic and phenotypic changes in this nematicidal Bt clone. Our genetic analyses had a special emphasis on prophages and transposases as they exhibit activity within the experiment and have been shown earlier to be significant drivers of adaptation (e.g., Chénais et al. 2012; Davies et al. 2016). Furthermore, plasmids, especially those that encode nematicidal Cry-toxins, are known to be important for virulence in this strain (Sheppard et al. 2016), thus we also focussed on changes and the loss of plasmids.

The sequencing of thousands of bacterial genomes has highlighted the fact that the majority harbours multiple prophages. In total, they account for up to 20% of the genome (Canchaya et al., 2003; Casjens, 2003), making them key actors in bacterial population dynamics (Fuhrman 1999; Brüssow et al. 2004; Geng et al. 2017). Our prophage analysis revealed eight prophage regions in the chromosome of the ancestral clone. To all of these, corresponding regions with only small sequence changes, mainly again in transposases, were identified in the chromosome of the adapted clone. Notably, it also revealed the acquisition of an incomplete 32.6Kb big prophage region in the adapted clone that could not be found in the reference chromosome. The new phage has apparently infected MYBT18679 within the experiment and subsequently been integrated as stable prophage into the chromosome.

The analysis of prophages on plasmids showed no major differences between the ancestral and the adapted clone. On plasmid 1 PHASTER identified five prophage regions (Table 3) of which the regions 2 and 3 as well as regions 4 and 5 were overlapping, respectively. However, experimental data indicates that both represent one single prophage. PHASTER is a database driven tool and thus performs poorly at identifying novel phages. Additionally, the authors mentioned that larger prophages could occasionally be split into a number of smaller prophages due to a paucity of BLAST hits (Zhou et al. 2011), this might be the case here as well.

Furthermore, we found massive transposase activity of nearly all annotated transposases within the chromosome; mainly of putative transposases for insertion sequence (IS) elements. Our results may indicate that the presence of the host represents a stressor for the parasite which increases transposase activity. Future studies need to document the baseline transposase activity to scale the strength of the change. However, McClintock (1984) suggested that transposable element activity could be a response to challenges to the genome and transposition of these small elements as a reaction to stressful conditions have been well-
documented for bacteria (Vandecraen et al. 2016). Different conditions such as UV light or sub-inhibitory concentrations of antibiotics in *Escherichia coli* (Eichenbaum and Livneh 1998; Lartigue et al. 2006), high temperature or oxidative stress in *Burkholderia* (Ohtsubo et al. 2005; Drevinek et al. 2010), radiation in *Deinococcus radiodurans* (Pasternak et al. 2010) or competence inducing conditions in *Bacillus subtilis* (Takahashi et al. 2007) were shown to promote transposition of IS elements. We suggest that the hosts’ immune system may similarly act as a stressor and caused this massive transposase activity. By serially transferring the cells to new, genetically diverse hosts, we increased selection pressure that enhances and triggers IS element activity additionally. Furthermore, several transposable elements can be mobilized by the passage to cell culture (Di Franco et al. 1992), which may serve as another source of stress.

To elucidate whether genetic changes also had an effect on the phenotype, we compared the ancestral and the evolved clone in two different phenotypic assays. The first one focussed on changes in the killing rate towards *C. elegans* and the reproduction of the host post infection. Reproduction was significantly higher in the adapted clone. In contrast, killing rate was drastically decreased in the adapted clone compared to the ancestral clone (Figure 2), what is in line with a previous study (Kloesener et al. 2017). This reduction in killing rate and the increased host reproduction indicate a reduction in virulence. MYBT18679 has previously been described as a nematicidal strain (Schulte et al. 2010; Masri et al. 2015) encoding two Cry-toxins that have been shown to kill *C. elegans* (Cry14Aa2 and Cry21Aa3). In general, Cry-toxins are activated in the insect gut where they bind to specific receptors and cause cell disruption and host death (Melo et al. 2016). The amino acid sequences of Cry-toxins have highly conserved as well as variable regions, the latter generating high host specificity (Palma et al. 2014). Toxins or complete plasmids on which toxins are located can be lost or exchanged between bacterial cells (Schulte et al. 2010; Masri et al. 2015; Sheppard et al. 2016). In this strain, two nematicidal toxins Cry14Aa2 and Cry21Aa3 are located on one single plasmid (plasmid 5, see Table 1) (Sheppard et al. 2016) that has been lost in the adapted clone (Table 1). We observed a massive reduction of virulence in the adapted clone which most likely results from the loss of plasmid 5. Nematicidal Cry-toxins represent as well an essential virulence factor in Bt (Masri et al. 2015) and a considerable metabolic burden. It has been shown earlier that Cry-toxins can get lost if the selective pressure to maintain these genes is reduced (Masri et al. 2015; Sheppard et al. 2016).

Interestingly, plasmid 6, carrying Cry34Aa5, Cry35Aa5 and Cry38Aa2 which target a complete different host range, was maintained in the adapted clone. Cry34Aa5 and Cry35Aa5 are known
to act as a binary insecticidal toxin against Lepidoptera, Coleoptera and Diptera (Schnepf et al. 2005; Kelker et al. 2014). Cry38Aa2, although sharing sequence similarity with characterised toxins, has so far not been shown to demonstrate toxic activity against a certain host (de Maagd et al. 2003; Baum et al. 2004). Although these three Cry-toxins have no nematicidal properties and are thus not needed under this selection regime, their genes were still maintained in this clone. Despite the fact that it has been widely demonstrated that plasmids can be costly for bacteria (Bouma and Lenski 1988), the gene expression is potentially more expensive than the replication and thus the carriage of the plasmid itself (Bragg and Wagner 2009; Lynch and Marinov 2015). Nevertheless, cells might maintain plasmids because they bear genes that increase their fitness (Bergstrom et al. 2000). Here, an explanation for this persistence could potentially be found in the evolutionary history of this strain. To date there has been no description of a Bt strain that has both, insecticidal and nematicidal properties. However, the transfer and acquisition of cry-toxin genes and their specific activities among Bt strains has been demonstrated soon after their first isolation (González et al. 1982). A reasonable explanation for our findings is that this strain might initially used an insect as host, leading to the need of stable insecticidal toxins as virulence factors, but gained the virulence towards nematodes more recently on an evolutionary time scale, e.g., via horizontal gene transfer (Thomas and Nielsen 2005). This would explain the easily loss of the plasmid by singularization of the strain when a host, so a selective pressure, is missing. Sheppard et al. 2016 reported a rapid loss of this nematicidal Cry-toxin encoding plasmid 5 from individual cells in MYBT18679 after ten weeks of serial passages on non-selective growth medium. Similarly, Bizzarri et al. 2008 observed changes in the chromosome and on plasmids of different Bt strains after repeated culture in a rich medium. We also needed to include growth steps during the isolation procedure of the parasites out of the dead worms and the subsequent generation of single clones. Thus it is possible that this non-selective growth of the strain combined with the instability led to the loss of this plasmid in our experiment. Furthermore, Sheppard et al. 2016 suggested that the loss of this Cry-toxin encoding plasmid could lead to a fitness advantage in the absence of a host. Cry-toxin crystals can make up to 25% of dry spore weight which cannot be invested in the production of offspring (Agaisse and Lereclus 1995). Although a host is present in our study, plasmid lacking and thus faster growing cells might had a selective advantage over plasmid carrying cells during exponential growth within the host and on plates in this experimental design. However, in a single infection, these plasmid lacking cells might face a dilemma in the following infection cycle, as they are not able to produce Cry-toxins to infect hosts anymore.
In contrast, plasmid 5 is always maintained at low frequencies within a population and never lost completely (Sheppard et al. 2016; Kloesener et al. 2017). This could be a hint for division of labour between the cells, where plasmid lacking cells are required for other tasks within the infection cycle and it might also explain why plasmid lacking cells persisted until the 10th host generation in this experimental setup. Plasmids 7-9 which are also not present in the adapted clone, have only few protein expressing genes compared to plasmids 1-4 (see Table 1) and none of these genes encode an obvious fitness advantage to the cell under conditions of host adaptation.

In the second phenotypic analysis we tested competitive ability of the evolved clone against the ancestral clone and against another Bt strain (MYBT18247). In Bt, inhibition of resource-competitors can for example be mediated by the production of bacteriocins (Abriouel et al. 2011) that usually display a high degree of target specificity against related bacteria (Tagg et al. 1976). Since the production of these inhibitory substances is metabolically costly and in many bacteria involves cell lysis for their release (Brown et al. 2009; Leggett et al. 2014), they should only be produced if competitors are present. The inhibition of the ancestral clone could thus be considered as an indirect measure for diversification, as bacterial clones are protected from the toxic effects by genetic linkage between the bacteriocin gene and an immunity gene (Riley and Wertz 2002). In line with our previous study (Kloesener et al. 2017) this clone did not start to inhibit the ancestral clone, indicating a low genetic divergence in terms of immunity or the recognition as a clone. Due to the fact that there was no other strain present in this treatment and no self-inhibition or stronger inhibition of MYBT18247 evolved, there was probably no selection for a higher competitive ability.

Our study gives new insights into the molecular genomic adaptations during a long-term challenge of a bacterial pathogen by a host organism. We identified treatment dependent evolved phenotypes that impact the key features of the virulent interaction of both, host and parasite. The analysis of the sequence data indicates that the dominating adaptation event of the underlying genotype is the activation of mobile genetic elements in all replicons of the parasite as well as the rapid loss of complete plasmids and a prophage. It shows the massive impact of the host on the parasite genome, e.g. in terms of plasmid loss and the consequences for virulence as a major fitness trait. Notably the impact of classical mutation events as SNVs and small INDELS is apparently not explaining the observed phenotypes.
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Author contribution

J.B. and R.D.S. designed the evolution experiment, J.B. and M.H.K. performed the evolution experiment. M.H.K. and R.D.S. designed the experiments for the phenotypic characterization of clones, performed data analysis and wrote the manuscript. M.H.K. isolated clones, performed phenotypic assays (killing rate, reproduction and inhibition) and analysed the corresponding phenotypic and genomic data. C.C did NGS mapping and SNV analysis. J. H. designed, supervised and performed NGS sequencing of the singularized adapted clone, wrote manuscript. H. L. processed and assembled NGS data, designed genome analysis strategy, performed genome annotation and comparative genomics, wrote manuscript. All authors read and accepted the manuscript.
Supplementary Information

**Experimental evolution**

- **General protocol**

This experimental evolution had a one-sided adaptation treatment where parasites adapted to a non-evolving host. After an initial infection with a single clone of the Bt strain MYBT18679, worms from the stock were subsequently exposed to Bt populations obtained from dead worms in every new infection cycle.

Prior to the start of the experiment worms of an outcrossed, genetically diverse population (Teotonio et al. 2012) were adapted to the laboratory conditions, aliquoted and stored in the -80°C freezer (note that they survive freezing, Stiernagle 2006). For the start of the experiment and throughout the experiment, worms were thawed and maintained on *Escherichia coli* OP50 inoculated Nematode Growth Medium (NGM) plates for few generations. The ancestral Bt clone was aliquoted and frozen after proliferation on NGM plates for 5 days. Prior to the exposure to nematodes, Bt was thawed, NGM plates (9cm diameter) inoculated with 2.5x10^6 Bt particles and incubated for 5 days to ensure that the majority of cells transformed into spores as only spores are associated with toxins that are the main virulence factors. 2 days before the exposure, worms were age-synchronised and purified of bacteria by alkaline hypochlorite:NaOH treatment (“bleaching”) that kills all nematodes and bacterial cells with the exception of nematode eggs (Stiernagle 2006).

Experimental evolution was performed in “worm balls”, transparent plastic balls with a diameter of 5cm, consisting of two halves, filled up with a layer of Peptone free Nematode Growth medium (PF) (Sicard et al. 2007). The worm balls force worms into constant contact with bacteria and subsequently minimize the escape behaviour of worms, which is relatively high in standard Petri dishes (Sicard et al. 2007). PF was used because it is unfavourable for bacterial proliferation, thus it ensures that spores cannot germinate into vegetative cells. *E. coli* OP50 was used as ad libitum food source to ensure sufficient nutrient supply for a whole week, thus they did not rely on Bt cells as food. Each worm-ball was inoculated with a total of 3.5x10^7 Bt particles and ad libitum *E. coli* OP50 in PBS. After inoculation, worm balls dried completely in a sterile bench before adding worms. Host populations were always started with ~150 L4 larvae one day after inoculation by washing off bleached worm maintenance plates with M9-buffer and pipetting a volume containing 150 worms to the worm balls.
Both antagonists were transferred to new worm balls once per week (equivalent to two host generations). Killing Bt was selected by collecting up to 30 dead worms from each worm ball to a PCR tube filled with 100µl liquid PF (i.e. without agar agar) at day two and three of exposure. After 2 days of incubation, during which bacteria could use up the host resources and sporulate, the bacteria were pasteurized (10 minutes at 70°C) to prevent unwanted coevolution of food or possible contamination. Next, they were plated on NGM (5cm diameter), incubated for 5 days, washed off with PBS and used for worm ball inoculation as described above. Worm populations from the stock (adaptation) were bleached as described above. After two days, 150 L4 larvae were transferred to the inoculated balls via pipetting. To avoid drift effects resulting from transferring only a part of the population, every second host generation 5% original genotypes of both antagonists were added. At each transfer (every second host generation), samples of both evolved antagonists were preserved. Evolved bacteria were frozen at -20°C and evolved worms at -80°C (Stiernagle 2006). The experiment was run at 18°C and 70% humidity.

Phenotypic assays

- **Competitive ability**

Inhibition of the ancestral MYBT18679 clone and a clone of the Bt strain MYBT18247 was tested in a plate assay. Prior to the experiment (24h) liquid overnight cultures were prepared with 100 µl Bt in a concentration of 10^7 spores/ml in 5ml LB and incubated in a rotary incubator (140 rpm) at room temperature. 100µl overnight culture of either MYBT18679 or MYBT18247 was added to 5ml warm soft-NGM (contains 79.5% less agar-agar than NGM), carefully shaken and spread over a Petri dish (diameter 9 cm) containing a basis of a thin, bubble-free PF-layer (peptone free nematode growth medium).

Bacteria that were tested for their competitive ability were added as spots of 10µl overnight culture onto the solidified soft-NGM surface. Each plate contained a spot of the ancestral clone, the adapted MYBT18679 clone, MYBT18247 and a negative control (LB medium). To avoid overlapping of the inhibition zones, all spots were separated by a distance of 23 mm and evenly spread over the plates.

Test plates were incubated for 5 days at 18°C and 70% humidity. A clearance zone around a spot on the agar surface indicated that the ancestral clone in the soft-NGM was inhibited by the clone in the spot. To determine the extent of inhibition as a proxy for competitive ability,
plates were scanned and spot size and clearance zone size were measured with ImageJ (version 1.43u) and subtracted from each other. The experiment was performed in 25 technical replicates.

- **Virulence (killing, reproduction)**

As a proxy for virulence we measured killing rate of worms by the ancestral and the adapted MYBT18679 clone towards ancestral *C. elegans* from the stock. Therefore 20 age synchronized hermaphroditic worms of the last larval stage (L4) were transferred into a worm-ball (diameter 5cm). Worm-balls contained a layer of PF-medium and were inoculated with $3.2 \times 10^7$ Bt particles and ad libitum *Escherichia coli* OP50 as a food source to prevent any effects caused by starvation. Prior to inoculation, Bt was grown for 6 days on nematode growth medium (NGM). Three days after exposure, the killing rate was measured as the number of dead worms divided by the sum of dead and surviving worms.

The same worm-balls were used to determine worm reproduction as a proxy for virulence. After the third day of exposure, worms were therefore washed off each ball using 1.5ml PBS. By counting the number of offspring in two subsamples of 10µl and calculating the mean, we determined the number of worms in 1ml that was collected from each ball in Eppendorf cups. Adult worms and their offspring could easily be distinguished by their differences in body size.

The experiment was performed in 19 technical replicates for both tested clones, respectively. Worm balls were stored at 18°C and at 70% humidity.

**Genetic analysis**

**Table S1:** Plasmid accession numbers in NCBI were simplified as following:

<table>
<thead>
<tr>
<th>plasmid abbreviation</th>
<th>NCBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>chromosome BTI679_contig000001</td>
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</tr>
<tr>
<td>plasmid 1 BTI679_contig000002</td>
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</tr>
<tr>
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<tr>
<td>plasmid 9 BTI679_contig000010</td>
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General discussion
General discussion

Parasites have a significant impact on their hosts and vice versa. Thus the continuous adaptation and counter-adaptation of host defence and parasite counter-defence can lead to a variety of phenotypic and genotypic changes in both antagonists. However, as multiple infections are common in nature (Read and Taylor 2001), parasites may interact with their coevolving host, but also with co-infesting parasites. Studies analysing not only the consequences of evolution between hosts and their parasites, but also looking at the effects for intraspecific interactions between co-infesting genotypes, are lacking. With a special emphasis on the parasite, this thesis focuses on both, on consequences of reciprocal evolution for the interaction with the host and on the effects for interactions with other genotypes.

The first chapter offers a comprehensive theoretical overview of the effects of multiple infections on virulence towards the host. Furthermore, it summarizes potential social interactions between the different co-infesting genotypes, the relevance of their relatedness and resulting consequences for virulence. The second chapter then presents empirical evidence for the importance of parasite-parasite interactions for host-parasite evolution. With the help of a selection experiment, two different strains of the microparasite Bt and its nematode host C. elegans evolved under controlled laboratory conditions in single infections. The consequences for the two strains after ten host generations showed strong individual differences in their ability to kill the host: whereas one strain massively decreased in virulence, it was not significantly affected in the other. However, in both strains the production of antagonistic substances towards the other strain was reduced, most likely as a result of the absence of other genotypes during infection. Nevertheless, both strains had in common, that coevolution with, or one-sided adaptation to a genetically diverse host led to rapid diversification of the clonal parasite population. These distinct clones had significant differences in virulence and antagonistic behaviour between each other, indicating that this infection can be considered as a multiple infection with distinct genotypes. The fact that antagonistic substances produced by some clones inhibited the ancestral clone, reflects the strong diversification over the course of evolution and gives a hint on potential parasite interactions within the host.

The last chapter presents the comparison of the ancestral parasite clone with an evolved clone from the evolution experiment on the molecular level. The aim was to analyse the underlying genetic mechanisms that caused the changes in individual clones presented in chapter two. By
using next generation genome sequencing we figured out the genetic factors that led to diversification within the clonal population. Furthermore, we highlighted the importance of bacterial genomic plasticity for adaptation: the results showed that changes were mainly caused by mobile genetic elements (MGEs), especially transposases and plasmids.

Taken together, in this thesis not only the theoretical background for host-parasite-parasite interactions is given, but also direct empirical proof for the relevance of intraspecific interactions between co-infecting parasites. Furthermore, it indicates that host-parasite coevolution may triggers the activity of MGEs and plasmids in the parasites' genome, what potentially enables rapid adaptation to the host, but also causes diversification between parasite genotypes. Diversification of clonal parasites likely affects within-host interactions between the different genotypes and subsequently also has consequences for host-parasite interactions. This experimental setup offered the opportunity for a comprehensive study of consequences of experimental evolution for the host (unpublished data from Joy Bose), for parasite populations and for individual parasite genotypes (Chapter II). Whereas empirical evidence so far largely came from bacteria-phage models, this setup enabled to present phenotypic and genetic changes for the evolution with a multicellular host. Furthermore, the distinction between host-parasite coevolution and parasite adaptation to a constant host, allowed disentangling phenotypic changes that were caused by adaptation from those that resulted from reciprocal coevolutionary interactions.

The results of Chapter II showed that phenotypic responses to host-parasite evolution were highly strain specific. Thus it is worth investigating more Bt strains to draw a broader picture for making general assumptions for phenotypic consequences of host-parasite evolution this system. Furthermore, as discussed in Chapter I, multiple infections by different parasite genotypes at the same time might change the outcome of host-parasite-parasite evolution drastically. Parasite-parasite interactions within the host can affect virulence, what is especially of importance for epidemiology, as many diseases are caused by multiple infections (Read and Taylor 2001). Further insight into the dynamics of multiple infections over the course of an infection could support the development of suitable treatments for patients. We also discussed, that not only the number of co-infecting strains is relevant, the ratio of the different strains might also have a strong influence. They are potentially crucial for the interaction between parasite genotypes and are thus also likely to affect virulence. Low ratios of a competitor might lead to the same dynamics as a single infection. But if virulence is dependent on the production of public goods as the Cry-toxins in Bt, host exploitation might be more efficient for a certain ratio of parasite genotypes as it is more challenging for the host immune
system. As Cry-toxins are frequently used in agriculture to kill insects damaging crops, a better understanding of these dynamics could improve their efficiency. It might help to extend the time span until hosts become resistant. Additionally, here we focussed only on co-infections by different parasite genotypes of the same species, but co-infections by different species are doubtlessly relevant as well.

This shows, that there is a lot potential for future studies to gain deeper insight into these complex dynamics in the *C. elegans* - Bt system. As the focus of the second chapter was on diversification from the ancestral clone, the analysis of antagonistic behaviour was performed against the ancestral clone. Additional tests between different evolved clones could provide more insight into interactions within the infected host. For future studies, a control population of both, the host and the parasite would be helpful, respectively maintained without the antagonist under the same conditions over the course of experimental evolution. By that, potential changes that are caused by the maintenance in the laboratory could be better disentangled from the changes due to the treatment.

**Conclusion**

In my thesis I present significant results on the rapid diversification of clonal populations of the microparasite Bt after experimental evolution with its multicellular host *C. elegans*. I was able to show, that host-parasite coevolution has tremendous consequences for the interaction with the host, but also influences the interactions between co-infecting parasite genotypes. I have further given hints on the importance of mobile genetic elements and plasmids for host-parasite evolution and the diversification within and between clonal parasite populations providing a basis for future research.
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Erklärung über die Eigenständigkeit der erbrachten wissenschaftlichen Leistung

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet.

Bei der Auswahl und Auswertung folgenden Materials haben mir die nachstehend aufgeführten Personen in der jeweils beschriebenen Weise entgeltlich/unentgeltlich geholfen.

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Ort, Datum                     Unterschrift