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Phage variation: understanding the behaviour of an accidental pathogen

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Understanding why carriers of meningococci occasionally develop invasive disease is a major challenge. Individual strains of meningococci are extremely variable and undergo dynamic changes in DNA content and organization. This heterogeneity of meningococcal populations might enhance the fitness of this human-restricted bacterium. The recent discovery of a meningococcal bacteriophage and its associations to disease is an intriguing example of this variability and could contribute to a better understanding of microbial commensal and virulence behaviour.

An accidental pathogen

The meningococcus (*Neisseria meningitidis*) causes life-threatening diseases, most importantly septicaemia and meningitis. The rapidity of onset, progression and severity of these infections is notorious. Despite this, the meningococcus is in most cases a commensal organism and acquisition of the bacterium normally leads to asymptomatic carriage. Why do meningococci occasionally manifest extreme pathogenic behaviour? Recently, Bille *et al.* [1] reported that meningococcal strains that are prone to cause invasive disease often harbour an M13-like bacteriophage integrated into their genomes. This novel observation adds another dimension to the challenge of unlocking the puzzle of meningococcal commensal and virulence behaviour.

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A complex pathogenic personality

It has been known for decades that the capsular polysaccharide of meningococci confers a measure of resistance to host immune clearance, whereas antibodies to the capsule promote killing and ingestion of the bacterium by phagocytosis [2]. These reciprocal fundamental insights into the susceptibility of humans to invasive meningococcal infections have been expanded in the past 20 years to produce a detailed, but evidently incomplete, profile of additional factors that determine the virulence and commensal behaviour of meningococci (Table 1).

What is special about this recent study? First, the approach embodies a bold and novel application of population biology and genomics. Second, the enhanced association of disease with strains that possess the phage apparently has a disproportionately greater impact on

Table 1. Molecular basis of meningococcal pathogenicity

Adhesins and invasins	Pilin and pilus-associated proteins (e.g. *pilC*)
	Opa proteins (multiple)
	Opc
	NadA
Pabulins[a]	Iron-acquisition proteins (e.g. Tbp1, Tbp2; LbpA, LbpB; HmbR)[b]
Evasins	Capsular polysaccharide
	IgA1 proteases
Toxins	Lipopolysaccharide

[a]Molecules involved in scavenging essential nutrients (in Latin, pabulum means food).
[b]Abbreviations: HmbR, high-affinity outer membrane haemoglobin receptor; Lbp, lactoferrin-binding protein; Tbp, transferring-binding protein.

young adults than on infants. If so, several factors and potential explanations must be considered and investigated further if the findings are to be adequately explained.

Heterogeneity of natural microbial populations: application of genomics and microarrays

In a previous study, it was pointed out that the virulence potential of meningococci is not uniform [3]. Based on polymorphisms in the DNA sequences of neutral housekeeping genes, it was shown using multilocus sequence typing that some meningococcal strain groupings (designated hypervirulent lineages) are more likely to cause invasive disease than are other strains (designated carriage strains). Can the genetic basis of this differing potential for disease be pinpointed? This question was approached using the completely sequenced genome (Z2941) of a meningococcal strain to construct a microarray that included 92% of the genes in Z2941. DNA from a representative collection of either hypervirulent or carriage strains could then be used as probes. Differential hybridization was used to investigate the presence or absence of specific DNA sequences. Excitingly, several distinct differences were found, the most striking being the presence of 8 kb of DNA in all the hypervirulent strains but in only a minority (10%) of carriage strains.

A novel meningococcal bacteriophage

To date, the gene content of the 8-kb element has not provided clues that might relate one or more novel functions to the association of the element with hypervirulent strains. However, based on the size, arrangement and homologies of its genes, the element was found to have characteristics of f1 or M13-type bacteriophages, including homology of one of its predicted open reading frames to *rstA* of CTX-Φ of *Vibrio cholerae*, an element that carries the genes for cholera toxin [4]. Indeed, further molecular characterization indicates that the phage can be integrated in the chromosome, that it can be excised and enter the bacterial cytoplasm as double-stranded circular DNA and that it can exit through a secretory pathway in a single-stranded (+ strand) state by a mechanism that implicates the pilus machinery possessed by meningococci. Other intriguing details include evidence of multiple chromosomal insertion sites, multiple copies of the phage per genome and integration, implicating the multiple copies of dRS3 repeats, of which there are hundreds in each meningococcal genome [5]. All of this suggests that the phage has evolved efficient mechanisms for interstrain spread. This is fascinating biology, indeed, but how does it help the understanding of the commensal and pathogenic behaviour of meningococci?

Virulence and transmission

Most obviously, one or more of the bacteriophage genes might be directly implicated in virulence: for example, enhancing host cell adhesion or invasion, or facilitating evasion of host clearance. This possibility can be investigated experimentally, although reaching unambiguous conclusions for the meningococcus is constrained by the lack of a satisfactory animal model in which to investigate the virulence of an obligate human pathogen. A different possibility is that the bacteriophage genes exert an indirect effect. For example, the insertion of a phage might exert a *cis*-acting effect by altering the transcriptional activity of flanking genes, or *in trans* through the production of proteins that affect the activity of genes that are unlinked to the 8-kb element. Virulence is complex and multifactorial, and probably involves combinatorial interactions. Although challenging, microarrays could be used to compare isogenic strains of meningococci (with and without the phage), with the aim of identifying consistently different patterns of gene expression. However, meningococci are known to modulate their commensal and virulence behaviour through intragenomic gene variation: for example, through phase variation of contingency genes [6]. Thus, the bacteriophage could modulate rates of phase variation through a *cis*- or, perhaps more likely, *trans*-acting effect that altered the patterns or frequency of phenotypic switching. It would be of interest to determine, for example, whether the phage-associated strains are mutators (or antimutators) or whether the phage disproportionately alters rates of phase variation in hypervirulent compared with carriage strains? These suggested mechanisms are the proverbial tip of the iceberg of possibilities but are tractable to investigation.

However, the data of Bille *et al.* [1] contain a subtlety. Recall that, in the single survey used in their study, the phage-associated disease isolates were ~30-fold more common in young adults (14–20 years) than in infants and young children (0–6 years). Thus, mechanisms of heightened pathogenicity, as outlined earlier, could contribute only a necessary, but not a sufficient, explanation of the observed findings. However, fitness increments associated with modulation of gene expression could swing the balance of behaviour of the microbes to facilitate pathogenicity, including the possibility that such virulence impacts less on the disease record of infants than of young adults.

What other lines of thinking might be used in generating hypotheses that could explain the effect of the phage-associated strains on the disease record of young adults rather than young children? Explanations based on heightened virulence might not be the only answer. As a different paradigm, let us suppose that the phage alters transmission characteristics. An increase in the acquisition rate of meningococci through enhanced transmission could explain an increase in invasive disease incidence and might provide a reasonable hypothesis for the findings, given that the relationship between carriage and disease incidence ought to consider acquisition rates of meningococci in the different epidemiological circumstances that are associated with the lifestyles of infants and young adults (see later). Note that, in this hypothesis, the role of the phage is not one that affects the intrinsic virulence (invasiveness) of the strain, but rather the probability of the occurrence of disease.

Hypothesis: enhanced transmission can increase incidence

We use a simple mathematical model to explain the underlying rationale of our hypothesis that a phage that

promotes transmission can increase the disease incidence without directly affecting the pathogenicity [7]. Consider a carriage strain with a transmission rate β, where invasive disease occurs with a (small) probability ε in which case no new carrier results. If the average duration of carriage is $1/\gamma$, a single carrier in a population with S susceptible hosts causes, on average, $S\beta\ (1-\varepsilon)/\gamma$ new carriers. This quantity is the reproductive number.

Enter the phage. If the phage were to increase the transmission rate to β', this strain would have a greater reproductive number than strains that transmit with rate β. But, according to epidemiological theory [8], in most cases strains with a greater reproductive number come to dominate the population. Hypervirulent strains tend to persist at low carriage rates [9], hence it is unlikely that they have an increased reproductive number. Moreover, the statistical properties of outbreaks of meningococcal disease indicate that hypervirulent strains have a slightly reduced reproductive number [7]. The reproductive number for phage-bearing strains must, therefore, be similar to, or slightly smaller than, that for carriage strains, and the increase in transmission must be balanced by a shorter duration of carriage. This hypothesis can be verified by longitudinal carriage studies.

How, then, do we explain the increase in the number of cases of meningococcal disease? Invasive disease normally occurs shortly after acquisition of the bacterium [10]. Therefore, the incidence rate of the carriage strain is given by $S\beta\varepsilon$, whereas it is $S\beta'\varepsilon$ for the hypervirulent strain. Because the hypervirulent strain transmits more efficiently, it causes invasive disease more frequently, without increasing the reproductive number (fitness): not only do hypervirulent strains turn over more hosts, they also do so more quickly.

How can a phage that has a slightly reduced fitness survive? Remember that the phage is well equipped for interstrain spread and can compensate for a reduction in fitness by horizontal transfer. To do so, it must encounter other phage-lacking meningococcal strains sufficiently often. This is the last piece of the jigsaw: other strains are encountered in hosts that have been colonized more than once. This is more likely to occur in young adults, who tend to have gregarious and more promiscuous lifestyles, than in infants, who are relatively quarantined. Therefore, strata of the population in which there is frequent transmission 'call' for phage, whereas there is selection against phage-bearing strains in strata with infrequent transmission. Similarly, one would expect selection for phage that enhances transmission.

Concluding remarks

Whether or not this hypothesis is correct remains to be proven, but it is feasible that possession of a prophage somehow alters transmission dynamics, the precise details of which remain to be uncovered. This highlights the urgent need for detailed study of the processes of carriage and transmission of meningococcal strains in natural populations.

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Genome Analysis

Extracytoplasmic function sigma factors in *Pseudomonas syringae*

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