Identification, evolution, and spread of bacterial virulence: consequences for genetic modification of bacteria.

Trudy M. Wassenaar

A literature study on bacterial virulence to evaluate the risk of genetic modification of pathogens commissioned by the Dutch Ministry of VROM (Volkshuisvesting, Ruimtelijke Ordening en Milieu).

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Rationale

The Dutch ministry 'VROM' has commissioned a literature research project entitled 'Pathogenicity and virulence of bacteria', the results of which will be used by the Committee on Genetic Modification (COGEM) to advise the ministry in a re-evaluation of the safety regulations for working with genetically modified organisms. VROM ordered an evaluation and summary of the scientific literature on bacterial virulence and exchange of virulence genes within bacterial populations (excluding plant pathogens). This work resulted in a report: 'Identification, evolution, and spread of bacterial virulence: consequences for genetic modification of bacteria' which contains 5 chapters:

- Chapter 1 gives a brief historical overview of how bacterial virulence was and is defined and assessed. A definition of virulence is proposed that enables the distinction between virulence genes, virulence-associated genes and house-keeping genes. The methods currently in use to determine virulence and to identify virulence genes were explored, and the functions of virulence genes for the bacteria that carry them were functionally classified.
- In Chapter 2 an inventory is given of current knowledge on bacterial virulence that was collected by searching public databases for described virulence genes. These literature data are sorted according to the functional classification of virulence genes.
- In Chapter 3 the role of the host in bacterial infections is explored.
- Chapter 4 describes the evolutionary origin and spread of virulence genes. Mechanisms for horizontal gene transfer between bacteria are summarized and examples of virulence genes that were obtained by such transfers are described. Experimental gene transfer by genetic modification is compared and contrasted with natural gene transfer.
- In Chapter 5 the results of this inventory are discussed and put into perspective. The possible risks involved with genetic modification of pathogenic bacteria is analyzed. Where appropriate it is indicated how future legislation could be based on new insights of origin and spread of bacterial virulence.

Chapter 1. Defining bacterial virulence

1.1. Pathogenic microbes as the causative agents of infectious diseases

In 1890 Robert Koch postulated guidelines to establish a standard for evidence of causation in infectious disease (based on early work by Henle): (i) the parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and clinical course of the disease; (ii) the parasite occurs in no other disease as a fortuitous and nonpathogenic parasite; (iii) after being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew. These postulates have been generally accepted for over 100 years with the possible addition that (iii) ... and the microbe can be re-isolated after experimental infection. See [1] for a recent review on the use of Koch's postulates in the past and at present times. It was Koch himself who recognized the limitations of these guidelines: he was unable to purify in pure form *Mycobacterium leprae*, as we are today. Not only the requirement of pure culture could not be met for many bacteria and viruses that live intracellularly; the lack of experimental models for human-specific pathogens further limited testing of the third postulate, and subclinical infection and carrier state colonization proved against the second postulate. The discovery of microbes that can produce distant injury by release of excreted substance, or employ immune mechanisms well after disappearance from the body, or cause cancer on a long-term basis, further weakened the applicability of Koch's postulates. Other complications were pathogens that require co-infection with a bacteriophage; host-dependent requirements (immunological status, physiology, genetic predisposition); and environmental risk-factors. Revisions of Koch's postulates were introduced to encompass these cases, in which immunological and/or epidemiological proof of causation was added [1].

With the development of molecular biological techniques, it became possible to identify the genes encoding virulence factors, and to identify genes of unknown function of which a possible role in virulence could be determined. This resulted in a new approach of research of microbial pathogenicity, in which the role of specific genes in (bacterial) virulence was the key point.

1.2. Molecular approach to bacterial virulence

The quest for virulence genes evolved together with the technical development of molecular biology and genetic modification of microorganisms. In the beginning of molecular microbiology, genes were identified that coded for virulence factors of known reputation. These virulence genes were then used as probes to look for analogies in other organisms. In a later phase the quest was reversed, and identified genes with unknown function were tested for their role in virulence. Nowadays the challenge is to filter out virulence genes from complete bacterial genomes. The application of molecular biology to microbial pathogenesis was put into words by Falkow [2] in a molecular form of Koch's postulates: (i) the phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species; (ii) specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence; and (iii) reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity. These molecular postulates posed new technical barriers: they require the possibility of genetic manipulation of the organism in question, and the availability of models to measure virulence. An alternative postulate was added in case genetic manipulation was not possible: (iv) the induction of specific antibodies to a defined gene product should neutralize pathogenicity. This addition is sometimes taken alone: when antibodies against a certain factor protect an animal from disease, this is sufficient to call this factor a virulence factor. Just as Koch recognized the limitations of his postulates, Falkow realized the implications of too rigid an application of his molecular postulates. For example, virulence can be a multi-factorial process, in which one gene can replace the function of another, so that multiple mutations are required for loss of phenotype. The classical view that fimbriae are essential virulence factors to uropathogenic *E. coli* was shaken by the finding that an adhesin present on the tip of fimbriae is responsible for the adhesion phenotype. However, although the adhesin in this example is the 'classical' virulence factor, it cannot function without the scaffold that the fimbriae provide, and inactivation of fimbrial subunit genes subsequently results in loss of virulent phenotype. By analogy, inactivation of genes involved in expression, processing, or secretion of virulence factors will also display loss of virulence phenotype. Thus, the new approach of 'molecular virulence' has resulted in the identification of novel

'virulence genes' that are not directly involved in virulence as such, but are indispensable to the organism for virulence because they are required for correct expression of virulence genes. Unfortunately in the literature there is no clear distinction between regulatory or structural genes that clearly have different functions in virulence [3]. All are described as virulence genes, because they all pass the tests described in the molecular Koch's postulates. At the start of this study an inventory was made of virulence genes as they were reported in the literature. These reported virulence genes were classified according to their role in virulence. It became apparent that there is a whole spectrum ranging from virulence genes to house-keeping enzymes that are all detected as 'virulence genes' when they have a function in survival in the host. This prompted for a more restricted definition of virulence genes than those genes that are detected in virulence assays, in order to correctly assess the risk of interspecies exchange of such genes.

1.3. Definition of bacterial virulence

There are many definitions of bacterial virulence, and virulence factors, in use, as summarized in Table 1 (after [4]). From the various definitions it can be recognized how the role of the host gained importance over the years. The definition that is chosen for virulence determines which factors would be included as virulence factors. As a consequence, the number of genes characterized as 'virulence genes' depends on the definition, as illustrated in Figure 1. Most investigators draw a line somewhere between circle 2 and 3 of Figure 1, since genes involved in basic cellular metabolism are regarded 'housekeeping genes' and are not generally regarded virulence genes. However, due to the methodology explained in section 1.4, such genes can be screened as virulence genes, since their inactivation can result in attenuation of virulence. For those genes for which a function in cellular metabolism is known, such results are interpreted as anomalous. For example, inactivation of the gene *AroA* results in attenuation but the gene is not considered a virulence gene because it is involved in aromatic amino acid biosynthesis, and as such is present in both pathogens and non-pathogens. However when no other function of the gene product is known, such genes are described in the literature as virulence genes. Another example of a 'housekeeping' gene with effect on virulence is the

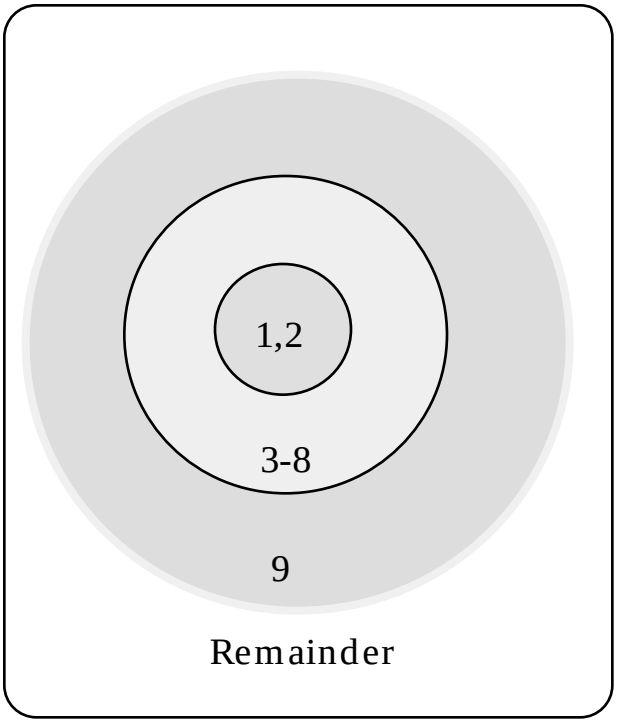
Table 1. Definitions of virulence and virulence factors used in the literature over time (for references see [4]).

Virulence is defined as:	**References Year** (taken from [4])	
property of invasive power	[5]	1914
Strength of the pathogenic activity	[6]	1927
relative capacity to enter and multiply in a given host	[7]	1934
Pathogenicity: the capacity of a microbe to produce disease	[8]	1949
synonym for pathogenicity: the capacity of a microbe to cause disease	[9]	1975
Degree of pathogenicity	[10]	1980
relative capacity to overcome available defenses	[11]	1983
percent of death per infection	[12]	1993
Disease severity as assessed by reduction in host fitness post-infection	[13]	1994
measure of the capacity to infect or damage a host	[14]	1997
relative capacity to cause damage in a host	[4]	1999
Virulence factor is defined as:		
Microbial products that permit a pathogen to cause disease	[15]	1977
a component of a pathogen that when deleted specifically impairs virulence but not viability	[9]	1980
A component of a pathogen that damages the host; can include components essential for viability including modulins	[4, 85]	1996

Mg^{2+} transport system of *Salmonella* [16]. The identified genes *Mgt*A/B are under *PhoP/PhoQ* regulation and are activated during invasion in vitro. Although mutants deficient in the genes for Mg^{2+} transport are equally invasive as wildtype bacteria in

vitro, they are avirulent in the mouse; illustrating how in vitro experiments do not always reflect the in vivo situation. In order to exclude housekeeping genes from the set of 'virulence' genes, the requisite is often added to Falkow's molecular postulates that virulence genes should not be expressed outside the host. This would exclude certain well-characterized virulence genes, for instance LPS-producing enzymes are expressed under all circumstances, and yet LPS is a generally accepted virulence factor. Moreover, lack of expression outside the host may be a reflection of the applied culture conditions. In conclusion, the border between virulence-associated genes and housekeeping genes is not sharp. All bacterial factors that have a function in establishing colonization and causing damage to the host, are included in the following definition of virulence-associated factors:

Figure 1
Depending on the definition of virulence more or less genes are called 'virulence genes'.



The number of virulence genes and virulence-associated genes included in a given definition are represented by concentric circles. In collection 1+2, virulence factors are directly involved in causing disease (class I and II from table 2). The addition of virulence-associated genes, including virulence regulatory genes and genes involved in secretion or activation of virulence factors (classes III to VIII from table 2) increases the number of identified virulence genes and thus the size of the circle (3-8). The gene pool identified by inactivation and phenotypic characterization (see Figure 2) includes all genes that lead to an attenuated phenotype, including certain house-keeping genes (circle 9). The remaining genes are remaining housekeeping genes, structural genes, and essential genes. The border between collection 9 and the rest cannot be exactly defined.

Definition of virulence-associated factors used in this study

The properties of pathogenic bacteria required for their lifestyle to survive, multiply, and cause disease in a host are: a capacity to compete with other bacteria in the host; to gain a foothold within a specific host; to avoid normal host defence mechanisms; to multiply once established; and in the cause of this process to produce damage to the host. Virulence-associated factors are all factors that are essential for this lifestyle.

A subset of virulence-associated factors in which house-keeping factors are excluded, are the virulence factors. Their genes (either directly coding for, or coding for their biosynthetic enzymes) are defined as:

Definition of virulence genes used in this study

All genes that encode for virulence-associated factors that are absent in apathogenic bacteria are defined as virulence genes. Their gene products are defined as virulence factors.

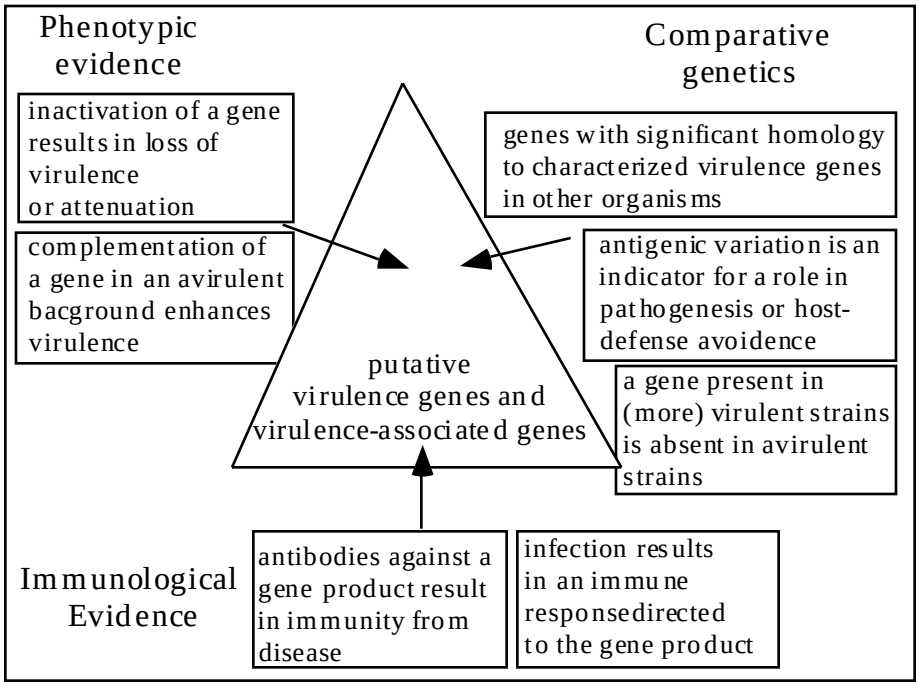
The use of this definition of virulence genes excludes those house-keeping genes that are involved in survival and multiplication in the host, when such genes are also found in apathogenic organisms. Virulence genes can be the structural genes for virulence factors, or encode the enzymes to produce such factors. It should be noted that many of the virulence-associated genes and their products (either directly encoded for or their enzyme products) are often reported in the literature as virulence genes.

1.4. Techniques to identify virulence genes

The molecular approach to study bacterial virulence has resulted in a number of techniques that are based on different principles (Figure 2). First, genetic methods are

used to obtain phenotypic evidence for virulence. As indicated in Figure 2, two approaches are used: (i) inactivation of a virulence gene must result in loss of virulence; or (ii) complementation of a virulence gene to a non-virulent strain must add virulent properties. Both principles are heavily dependent on models to determine virulence. Models to determine virulence are ideally animal models that minutely mimic natural disease, but more often animal models are used that display only some of the naturally occurring characteristics, or in vitro models that only poorly resemble disease characteristics. Most processes leading to virulence are multi-factorial and stratigraphically organized. The complicated interaction of host and bacteria is often ignored when in vitro models are applied (for better or for worse). Even under simplified conditions of in vitro models a presumably straightforward process such as bacterial invasion can be driven and regulated by multiple genes and gene loci, which work in concert or complementary. Inactivation of one link of the chain may eliminate invasiveness, but complementation in a different setting may require several genetic loci.

Figure 2.
Different approaches to identify virulence gnees and virulence-associated genes



Alternatively, inactivation of a factor may be overcome by alternative factors so that loss of virulence is not observed, but complementation in a different environment may have strong phenotypic effects. The relevance of the applied models to extrapolate their outcome as phenotypic evidence of virulence is a point of debate, which is pragmatically ignored by lack of an alternative.

A second approach for identifying virulence genes is based on the proposed immunogenicity of virulence factors (see Figure 2). Knowing that acquired immunity can protect against disease, it is assumed that protective antibodies are directed against virulence genes. As a consequence, an infection should result in an antigenic response that is directed against virulence factors. A third approach is to collect data from comparative genetics which will be treated in detail in section 1.5. In addition to these approaches, several techniques have been developed to identify and characterize bacterial genes that are induced during the intracellular infection and, potentially, play a role in pathogenesis. Two recent papers review current methods [17, 18], of which 'In Vivo Expression Technology' was one of the first [19].

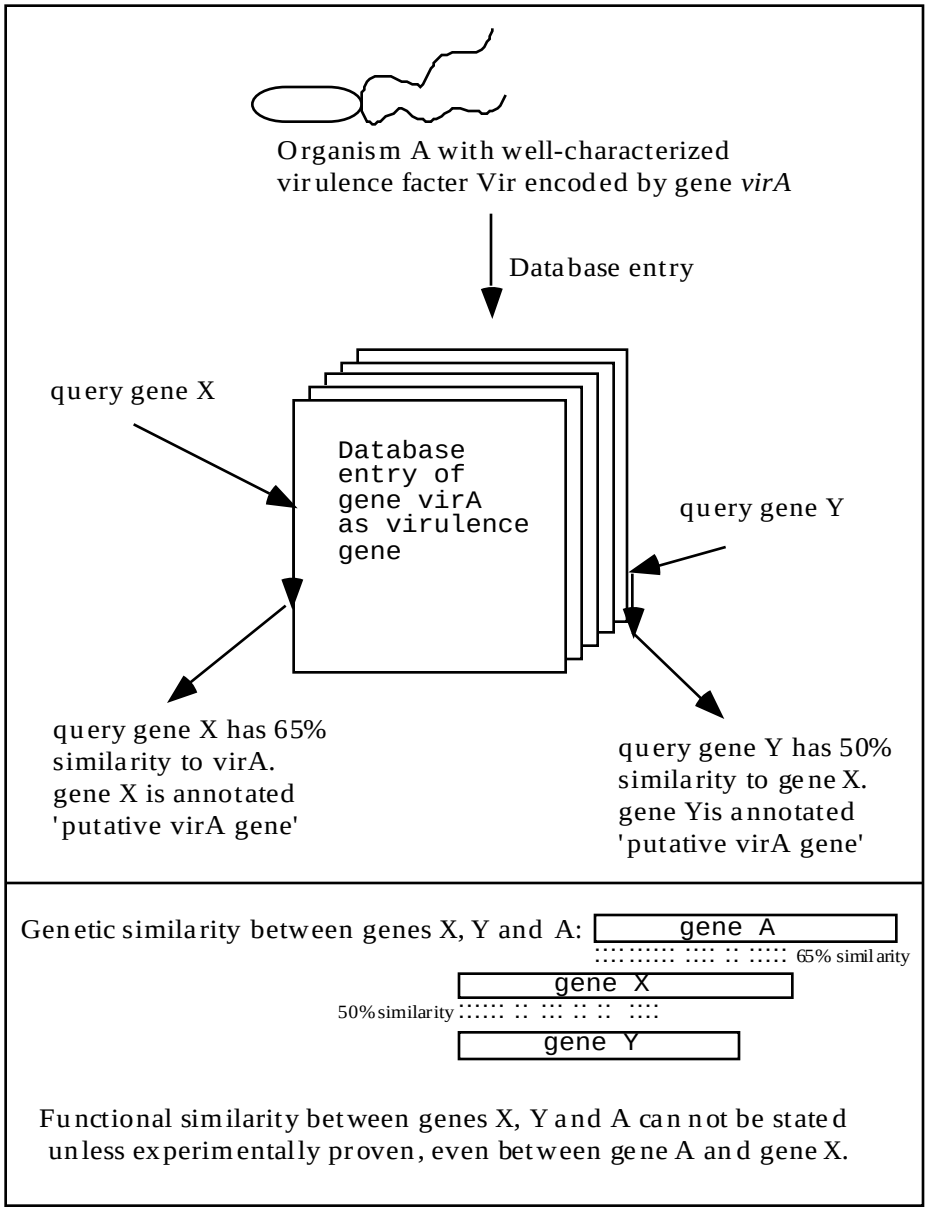
Ideally, for the identification of virulence, several approaches should lead to the same gene or set of genes, and a virulence gene should have more than one characteristics from Figure 2. Even then, the controversy between housekeeping genes and virulence genes is not always solved. In the example given above (section 1.3), the housekeeping magnesium transport system of *Salmonella* is under *PhoP/PhoQ* regulation, and is activated during invasion in vitro [16]. Another example is glutamine synthetase of *Salmonella typhimurium*, which is under the regulation of NtrC (an alternative sigma factor that can be indicative for in vivo regulation of expression) and which was identified as a virulence gene based on phenotypic evidence, since inactivation resulted in attenuation [20]. The enzyme presumably provides glutamine to the organism while surviving in the host, and could for that reason be considered a virulence-associated gene that enables colonization. Since glutamine synthetase is also present in apathogenic bacteria it is not considered a virulence gene here. In the approach applied here, the absence of virulence genes in apathogenic bacteria receives a lot of weight. Two points need to be considered here: (1) the outcome of comparative genetics is heavily dependent on the content of the databases used; and (2) gene function is not always correctly

predicted as demonstrated in the next section. Putative virulence gene candidates should therefore at least be confirmed by phenotypic evidence, despite the mentioned shortcomings of such evidence.

1.5. Future trends in virulence gene identification

Comparative genomics is now feasible for bacterial pathogens: complete genome sequences can be compared by in silico subtractive hybridization. With the expanding annotation of genes from genome sequences, this can lead to the identification of new virulence genes [20b, 20c]. The annotation of newly sequenced genes that are now generated in vast quantities by high-throughput genome sequencing projects is based on sequence identity. The identification of virulence genes by comparative genetics, based on genetic similarity is risky for two reasons. First, an acceptable level of sequence conservation is seen as (indirect) evidence of conserved function, so that the gene function of a newly sequenced gene is extrapolated from a well-characterized analogue in another species. However, genes may have a niche-adapted function in a particular organism, and this may have its reflection on the role in virulence. Therefore, even a high degree of genetic conservation should be experimentally tested before functional conservation can be assumed. Second, sequence similarity searches have resulted in a new phenomenon in virulence gene identification for which the term 'putativism' would be appropriate, and which is explained in figure 3. Mis-annotation based on 'putativism' is quite common, since it is now easier to generate sequencing data than to experimentally prove a function of the given gene product. An example of ambiguous use of data base annotations is a publication [21], where a newly discovered protein-tyrosine kinase gene in *E.coli* was used to screen public databases, and similarity to LPS-producing or exporting genes was detected. Since LPS is a known virulence factor (or, more correctly, a virulence-associated factor), a role of the discovered tyrosine kinase gene in virulence was postulated, without further experimental evidence. Two other pitfalls of comparative genetics are facing opposite directions. On the one hand different genes that share no sequence homology can have identical functions, as is demonstrated for *act*A of *Listeria monocytogenes* and *ics*A in *Shigella flexneri* whose gene products recruit host cell actin

Figure 3
The pitfalls of comparative genetics in using database annotations: how 'putativism' is spreading through databases



[discussed in 22]. This function similarity will go unnoticed by genome comparison. On the other hand, sequence homology does not always predict function, as illustrated by a calmodulin gene in *Saccharomyces cerevisiae* that can still be fully functional when the calcium binding domain is lost [22].

A second approach to predict virulent properties of a gene by comparative genetics is to look for regulatory or export signals that are known to be involved in regulation of virulence. For instance, by comparison all autotransporters (virulence-associated factors) share certain structural domains for secretion [23]. The recognition of such domains can aid to identify novel virulence (virulence-associated) genes. Knowing that many virulence genes are under RpoS regulation, a strategy was set up to identify novel genes regulated by this alternative sigma factor [24]. Again, the role in virulence of genes identified in this way remains to be proven, since even metabolic enzymes can share their regulation with virulence genes [25].

As indicated in Figure 2, an alternative approach to identify novel virulence genes comes from the observation that many virulence genes display antigenic polymorphisms, presumably to evade the selection pressure of the host immune system [26]. The correlation between polymorphism and virulence is so strong that polymorphisms are indirect evidence for a role in virulence. Unfortunately the term polymorphism is used for different phenomena. On the one hand the term is used when one isolate of a bacterial species can produce antigenic variants of a gene product by means of gene multiplication, alternative expression, or post-translational modification. On the other hand polymorphism is used for antigenic or genetic differences observed between isolates of the same species, for which the term allelic polymorphism is more exact. In addition, slippage during replication or translation can cause variation in the number of DNA repeats (with units of 1 to 7 nucleotides) present within a gene, leading to polymorphic offspring (either represented in DNA or in protein) of a given cell [27]. The result of the latter polymorphism is phase variation: a binary expression switch of that gene (which is then called a contingency gene). Some bacterial species have many such contingency genes in their repertoire. The resulting exponential combinations of expression profiles gave these bacteria their name 'quasi species' [28]. All of these polymorphic mechanisms serve the general goal of adaptation to varying conditions; in the case of pathogens this is often, though not exclusively, a mechanism to avoid host defense responses. With the high throughput of sequencing data, it becomes possible to identify putative virulence properties for genes based on the polymorphic nature of their predicted translation products. For instance, the genome sequence of *Haemophilus influenzae* enabled the

identification of novel virulence genes by searching for repeat sequences that are known to be sensitive to slip-strand replication [29]. In the future it may even become possible to predict posttranslational modification of predicted open reading frames, which may have an effect on virulence properties (for instance variation in glycosylation resulting in antigenic variation) but at present this cannot yet be predicted from genome sequences.

1.6. Classification of bacterial virulence factors and virulence genes

From the former sections it will be clear that there are many ways of defining, identifying, and testing virulence genes. Since each pathogen has evolved to fit its own niche, they do not share common pathogenic characteristics. Despite the recognition of common themes in bacterial virulence ([30-32]), a larger part of all virulence genes described in the literature that resulted from over 30 years of research have little in common, other than having some function in virulence. In order to interpret the vast amount of data on this subject these genes need to be classified. In the context of this study a classification of virulence genes is proposed, in which the role of the virulence gene in pathogenesis and life-style of the bacteria is used as the key feature (Table 2). In Chapter 2, examples of virulence factors collected from the literature are classified according to this proposed classification.

1.7. Is the perspective of virulence genes correct?

Different paths lead to the identification of virulence genes. (Figure 4). Every pathogen requires a diverse and unique set of genes to allow it to cause disease. In an approach that could be characterized as 'top down', the phenotypic characteristics of a pathogen ('invasive', 'toxin producing', 'phagocytic survival') is nailed down to bacterial factors responsible for these phenotypes, and subsequently the coding genes for these factors are characterized. Zooming in on the individual role of each of these genes is essential to understand the complex mechanisms behind disease. In a 'lateral' approach, the fact is used that pathogens often employ similar pathogenic mechanisms, so that analogies

Table 2. Proposed classification of virulence genes.

Class	Description	Examples *
I	Primary virulence factors	toxins, invasins
II	Primary colonization factors	adhesins, fimbriae
III	Auxiliary virulence-associated factors.	flagella, stress-proteins
IV	Auxiliary colonization factors (virulence-associated factors)	iron-uptake, urease, neuraminidase
V	(pre-) inflammatory stimulus factors	modulins
VI	immune system evasion factors and bacterial defence factors	surface layers, IgA proteases, toxins
VII	Regulatory genes of expression of virulence factors (virulence-associated genes)	Sigma factors, response regulators
VIII	Export, activation, and processing systems for virulence factors (virulence-associated factors)	secretory mechanisms, chaperonins

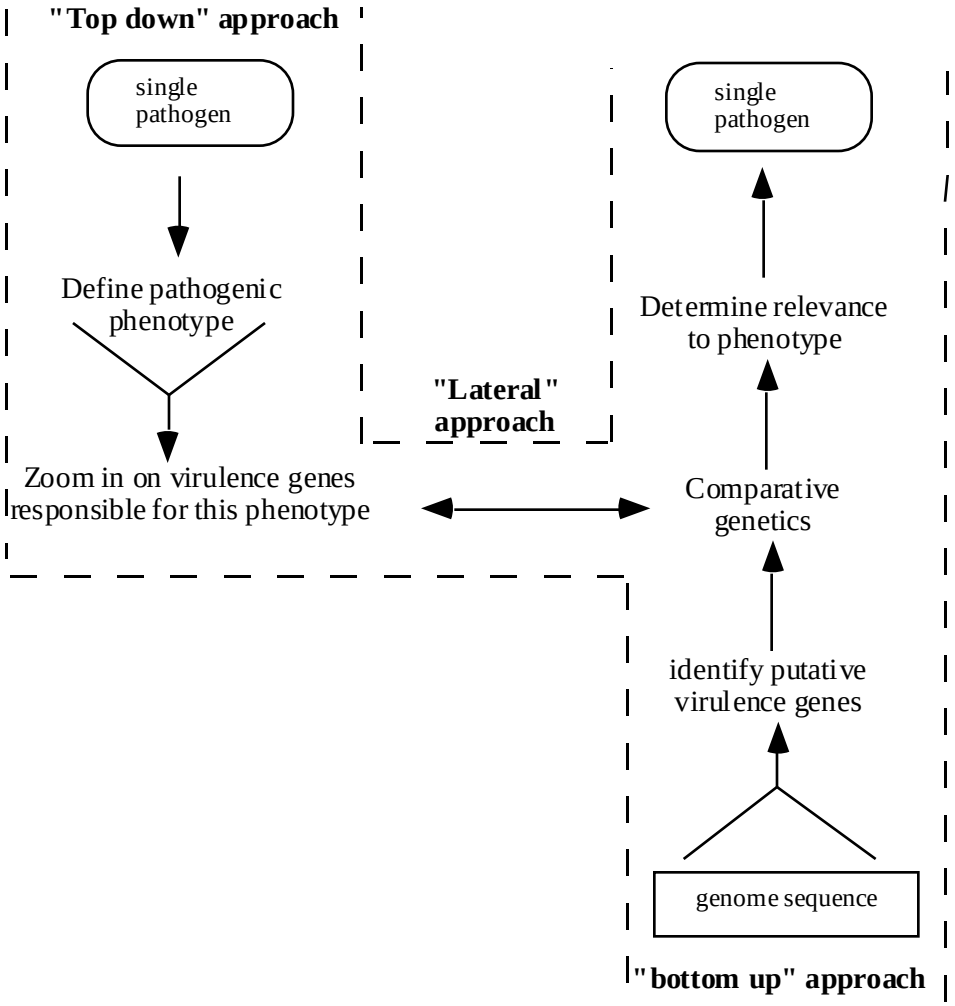
* Not all given examples apply to all pathogenic microorganisms. See Chapter 2 for further explanation.

between virulence factors can be applied for identification strategies. In parallel, genetically related organisms that have a different pathogenic repertoire can be compared to identify the genes responsible for the differences in virulence. Since a few years, the 'bottom up' approach of genomic sequencing has become available, where gene function is predicted from sequences and comparative genetics can be applied to complete genomes. In addition the role of the host and his immune status needs to be considered (Chapter 3). All of these paths have resulted in a pool of genes that share the common denominator 'virulence gene', but often have little else in common, as will be illustrated in Chapter 2. Each pathogen has evolved a pathogenic strategy combining one or more mechanisms that operate in a concerted manner.

This is demonstrated by *E. coli* O157:H7, which produces shiga toxin, in addition contains the attaching and effacing pathogenicity island that is responsible for adherence,

Figure 4.

Different paths lead to the identification of virulence genes



and also a hemolysin operon [33]. The shiga toxin is probably the primary cause of intestinal and systemic lesions, and was named after *Shigella dysenteriae* where it was first isolated. The *eae*-genes that are responsible for attachment are also found in enteropathogenic *E. coli* that lack the shiga toxin. The hemolysin locus is highly homologous to that of other enteropathogens (*E. coli* and *V. cholerae*). The combination of shiga toxin, hemolysin, and the *eae* locus results in the hemorrhagic colitis typical for O157:H7. The different components of this virulence strategy result in other bacteria in a different pathology due to a new combination of virulence components. The effects of

such combinations will become apparent as more genome sequences of virulent bacteria become available.

Our view on bacterial virulence factors is biased because of the experimental setup applied [33b]. For instance, many bacterial toxins are described as hemolysin, because their toxicity to erythrocytes is easy to determine. However, the bacteria that produce these compounds may, in real life, never encounter erythrocytes, and their toxins may be targeted at leukocytes or other host cells instead. This distinction may seem irrelevant, however the classical role of hemolysins is thought to be release of heme as an iron source, and as such the term hemolysin (and the hemolytic assay) is often erroneous. This is just one example of how our perception of bacterial virulence factors is influenced by experimental design. Ideally, experimental shortcomings, subjective observations, and the anthropomorphic view on pathogenicity (as discussed in Chapter 5), should all be considered when establishing the relevance of a certain virulence gene to the pathogenicity of a microorganism.

Chapter 2. Inventory of publications on bacterial virulence factors.

A database of scientific literature (Medline) of the last 10 years was searched for bacterial virulence factors and bacterial virulence genes using the terms 'bacterial' AND 'virulence' AND 'gene' OR 'factor'. Although this search does not cover all available literature, it is sufficient to get an overview of our current knowledge of bacterial virulence and to indicate in which directions research of bacterial virulence factors is moving. It should be noted that the publications are biased for virulence factors that are easy to determine (especially colonization factors), and those that have received more attention because of their potential as vaccine candidates or because certain topics became 'fashionable'.

Of the 818 publications found within the search 480 were analyzed, and 160 were classified, based on the content of the abstract, into the classes as proposed in Table 2 of Chapter 1. The division between primary and auxiliary virulence factors, or primary and auxiliary colonization factors, is made to distinguish virulence factors (as defined in section 1.3) from virulence-associated factors.
Only papers in English were included. Papers describing methodology or virulence without the identification of the responsible factors or genes were discarded. Duplicate findings were ignored. In general classification of virulence factors proposed in section 1.6 proved useful to categorize the literature. In some instances publications described properties belonged to more than one class because virulence factors may display multiple functions (e.g. involved in adhesion as well as invasion, toxins that are also inflammatory stimuli, colonization factors that also help evade the immune system, etc.), or because papers addressed several problems. Below follows a short description of virulence factors ordered according to this classification. The extensive list illustrates the diversity of virulence factors, however for continuation of this treatise on general features of bacterial virulence the reader can skip the next sections and proceed to the last paragraph of this chapter.

I. *Primary virulence factors*

In several bacterial infections it is obvious which bacterial factor is the primary cause of virulence. This is mostly true for exotoxins, like enterotoxins produced by enteropathogens [32], or botulin toxin produced by *Clostridium botulin.* Although toxin-producing bacteria that lack colonizing properties are strictly speaking not included in the definition of virulence, they are included here because their toxin genes can be regarded as virulence genes. Invasins are essential for invasive organisms, however once bacteria are inside macrophages, an array of factors is required to survive and multiply in this microenvironment. The factors involved in these processes were classified as auxiliary virulence factors, or factors produced to evade the immune system of the host (see below), because they represent properties that are required for the life-style of the intracellular organism. Examples of toxins and invasins found in the recent literature are:

• Toxins

-Many enteropathogens produce toxins that are the primary cause of enterocyte malfunction. *Vibrio cholerae* and enterotoxic *E. coli* produce cholera toxin which activates adenylate cyclase, which by Ca^{2+} efflux results in loss of the membrane potential of enterocytes [32]. *Shigella dysenteriae* and shiga-toxin producing *E. coli* produce shiga toxin (also called verotoxin) which inhibits protein synthesis [33].

-*Bordetella* spp. produce an adenylate cyclase toxin that inserts it's active component into the host cells [34].

-*Vibrio parahaemolyticus* produces a heat-stabile hemolysin that destroys enterocytes [35].

-An exotoxin produced by *Arcanobacterium* (*Actinomyces*) *pyogenes* fulfills all molecular postulates of a virulence factor [36].

-A metalloprotease secreted by *Vibrio vulnificus* produces similar skin lesions as the bacterial infection does [37].

-Pneumolysin is an essential toxin for *Streptococcus pneumoniae* virulence [38, 39].

-Proteases produced by oral pathogens (e.g. arginine-specific cysteine proteinase of *Porphyromonas gingivalis*) causes tissue destruction and impaired immune responses [40, 41].

• Invasins

-Invasins, intimins, and internalins are described for invasive *E.coli*, *Salmonella* and *Yersinia* spp. [32, 42].

-Opacity protein adhesin of *Neisseria meningitidis* binds to cell surface heparan sulphate proteoglycans as a first essential step to invasion [43].

-by means of sequence similarity, internalins were isolated in *Listeria ivanovii* using probes from *L. monocytogenes* [44].

• Combination strategies of toxins and invasins

-*E. coli* O157:H7 is invasive and produces shiga toxin and hemolysin [33].

-*Listeria monocytogenes* produces lysteriolysin (a hemolytic cytotoxin), phospholipase C and the membrane protein ActA, which are essential for intracellular survival, escape from the vacuoles and spread of the organism, and are expressed depending on the compartmentalisation of the organism [45-49].

• Reported virulence genes that do not fit the definition used here

-An example of a 'toxin' that is re-classified as a virulence-associated factor here, because it is also produced by apathogenic bacteria, is peptidoglycan. For a number of organisms, including *Treponema denticola* [50], a cytotoxic effect of peptidoglycan was demonstrated on epithelial cells. However, since peptidoglycan is a structural component of bacteria, it is not included in the definition of a virulence factor.

-Under certain conditions, opportunistic pathogens can cause damage to their host. The factors by which they do so are not included in the definition for virulence genes. For instance, invasion is a key factor to virulence of oral commensals like *Prevotella intermedia*, and toxins are also produced that specifically target for leukocytes [51, 52]. However these bacteria are considered normal mouth flora in the majority of cases.

II. *Primary colonization factors.*

Those bacterial factors that interact directly with host tissue to enable contact and thus colonization are regarded primary colonization factors here. Fimbriae can adhere to mucosal surfaces by their structural units, or by adhesins that are carried at the tip. A second class of colonization factors are enzymes produced by the bacteria that are essential to colonize the particular niche, however most of these are virulence-associated

factors since such enzymes can also be found in apathogens. Examples found in the recent literature:

• Adhesive structures

-The attaching and effacing genes of enteropathogenic *E. coli* are required for the typical bacterial microcolonies that intimately adhere to the surface of tissue culture cells, with accumulation of cytoskeletal actin under the bacteria [53, 54]. Intimin is also required but not sufficient [55].

-The type-4 pilus of *Neisseria gonorrhoeae* is a dominant surface antigen which facilitates adhesion to host target cells, an essential event in gonococcal infection [56].

-Pneumococcal capsular polysaccharide enables attachment and colonization in the middle ear resulting in otitis media [57].

-The fibronectin-attachment protein enables *Mycobacterium avium* to enter the host through the gastrointestinal tract [58].

-*Streptococcus* spp. produce adhesins that bind to blood group P-related disaccharide that appears to be essential for causing septicaemia and meningitis [59].

-A *Proteus mirabilis* adhesin present on fimbriae was discovered by similarity to *E. coli* adhesin [60].

• Essential colonization enzymes that are virulence-associated factors but not primary colonization factors:

-Urease produced by bacterial pathogens in either the urinary tract [61] or the gastroduodenal region act as a colonization factor. Urease is also essential for *Helicobacter pylori* for neutralizing the gastric pH [62]. However, urease is an enzyme present in apathogenic bacteria so it is classified a virulence-associated factor. Similarly, glutamine synthetase is required for intracellular survival of *Salmonella* [63], but it is regarded a virulence-associated factor (see below) and not a primary colonization factor.

III. *Auxiliary virulence factors (Virulence-associated factors)*

-Motility by flagellar activity has been implied in virulence of several enteropathogens, presumably to reach the preferred site of colonization and to overcome peristaltic movement. For a number of organisms impaired virulence was demonstrated for mutants

in flagellar biosynthesis or rotation [64, 65]. Since apathogenic bacteria can also be flagellated, these are considered virulence-associated factors.

-A protease-collaginase gene was identified that increased virulence of *Proteus mirabilis* by signature-tagged mutagenesis [66].

-Specific antibodies against Hsp60 (a GroEL analogue with chaperonin activity) of *Legionella pneumophila* were used to demonstrate a role of this surface-exposed protein in attachment and invasion [67]. a GroEL analogue of *H. pylori* is required for activity of urease [68]. Without such chaperonins, the pathogens could probably not survive their niche. Since GroEL is present in all bacteria, it is considered a virulence-associated factor here.

-Phospholipase C produced by *Bacillus cereus* caused tissue destruction and induced matrix metalloproteinase production in epithelial cells which adds to the pathology [69].

-*Pseudomonas aeruginosa* PA-I lectin contributes to the respiratory epithelial damage during respiratory infections, as do glycolipids produced by this organism [70, 71].

-A role in virulence for protein tyrosine kinases is assumed because of a correlation between expression of these enzymes and virulence, however molecular evidence is lacking [72].

IV. *Auxiliary colonization factors (Virulence-associated factors)*

-Factors required for iron uptake and heme-utilization, e.g. transferrins, hemoglobin protease, hemolysins, and siderophores [73-76] are regarded virulence-associated factors, that are required for the specific lifestyle of the organism. The above mentioned examples of urease and glutamine synthetase are also auxiliary colonization factors, and as such, virulence-associated factors for a number of organisms.

-Superoxide dismutase protects Gram-negative bacteria from exogenous oxidative damage, for instance during intracellular survival of *Salmonella* in macrophages [77, 78]. In these organisms the enzyme plays an important role in evasion of macrophage killing, however superoxide dismutase is a house-keeping enzyme in apathogenic bacteria and is thus classified as a virulence-associated factor.

-Stress proteins (like GroEL and other heatshock proteins) are required for the adaptation to the hostile environment that bacteria encounter, especially when dealing with macrophages [79].
-The bundle-forming pili of enteropathogenic *Escherichia coli* are implicated in the formation of complex, three-dimensional colonies via bacterium-bacterium interactions [80, 81]. These pili help establishing colonization but are not directly involved in interaction with the host. For that reason they are excluded as primary virulence factors.
-Neuraminidase (sialidase) is essential to *Haemophilus parasuis* for providing nutrients [82] but can also be found in apathogens.
- *Prevotella bivia* is an example of an opportunistic pathogen. It produces sialidases that destroy mucins during bacterial vaginosis and by doing so may enhance adherence of other bacteria [83].
-Inhibitors of proteinases protect *Streptococcus pyogenes* against proteolysis [84].

V. *(Pre-) inflammatory stimulus factors (virulence-associated factors)*
Bacterial virulence factors have been grouped by others as adhesins, aggressins, impedins, and invasins (these groups would fall in classes I and II of the classification used here)**.** By analogy to these groups, the array of bacterial cytokine-inducing molecules described for pathogenic bacteria could be called "modulins" [85], because the action of cytokines is to modulate eukaryotic cell behavior. Since modulins affect the inflammatory response of the host, they are classified here in class V. For instance, *Mycoplasma* alters inflammatory responses by mediating secretion of pro inflammatory cytokines (TNF alpha, IL-1, and IL-6) [73].
• Most reported 'virulence factors' within this class are virulence-associated factors that are present in pathogens as well as non-pathogens:
-Gram-negative bacteria causing septic shock cause overproduction of TNF-alpha by means of their LPS [87]. Gram-positive bacteria causing septic shock use TNF-alpha independent mechanisms by means of cell-wall components [88, 89]. In both cases overwhelming numbers of bacteria must be present for the pathology. A breakdown in host defense is probably the trigger of most septic shocks, and this disease cannot be regarded as a specific property of certain pathogens. LPS is a potent immunostimulatory

molecule which activates the innate host defense system [89-91]. Recent data indicate that bacteria can regulate their lipid A (a component of LPS) in response to different host microenvironments [92]. In murine *Salmonella* infection death is directly dependent on the toxicity of lipid A [93]. A *Haemophilus ducreyi waaF* mutant is less virulent since it's LPS induces an inflammatory response in skin [94]. A lipid A-associated protein of *Porphyromonas gingivalis* (an opportunistic pathogen) stimulates IL 6 synthesis [91]. Although in these examples LPS mutants are less virulent, LPS should be regarded as a virulence-associated factor and not as a virulence factor because it is also present in apathogenic bacteria.

-Lipoproteins (which can be classified as house-keeping or structural components) are released from growing *Enterobacteriae* and this released lipoprotein induces cytokine production and pathologic changes associated with gram-negative bacterial infections [95].

VI. *Immune system evasion factors and bacterial defense factors*

Many bacterial strategies have evolved to evade the host's immune system or to defend the bacteria against efficient killing. Some strategies are applied by a number of organisms, others are highly species-specific:

-Crystalline surface layers of different bacteria prevent opsonisation of bacteria by antigenic variation though the molecular mechanism for this variation varies among species. Capsular polysaccharide is a major determinant of serum resistance for a number of Gram-negative bacteria, and is a major determinant for causing meningitis [96, 97].

-*Mycoplasmas* have developed various genetic systems providing a highly plastic set of variable surface proteins and lipoproteins to evade the host immune system [98].

-*Streptococci* produce M protein that inhibits phagocytosis and which contains a surface-exposed hypervariable region that prevents complement attack by binding a plasma complement inhibitor [99, 100].

-Slime polysaccharide, and lipopolysaccharides, such as produced by *Shigella* spp. renders resistance to serum killing and phagocytosis [101-103]. A similar role has *Streptococcus suis* capsule [104]. Glycocalyx from *Bacteroides* [105] and *Staphylococcus*

[106] inhibit the chemoluminescence and chemotactic responses of PMNLs without being toxic for PMNLs.

-IgA proteases produced by some bacteria limite effective mucosal immunity [107]. Proteinases with a narrow specificity can specifically degrade collagen and IgG [108]. Many pathogenic bacteria possess cell surface receptors which can bind immunoglobulins via the Fc portion that render these Igs ineffective [109]. It could be argued that these factors are colonization factors, however since they have a distinct function in evasion of the host's immune system they are classified as VI.

-*Yersinia pseudotuberculosis* and *Yersinia enterocolitica* inhibit NF-kappa B and TNF-alpha cytokine production to limit or inhibit inflammatory host response. *Yersinia* contains a virulence plasmid which encodes a factor impairing the normal TNF-alpha response of infected macrophages [110-113]. *Brucella* spp. do the same by an unidentified mechanism [114].

-Cytotoxic necrotising factor type 1 from pathogenic *E. coli* induces a decrease of PMN transepithelial migration and thus protects the bacteria from destruction [115].

-*Salmonella* can induce apoptosis in macrophages by unidentified virulence factors that depend on secretory pathway III and is regulated by growth phase of the bacteria [116].

- A *Pseudomonas aeruginosa* quorum-sensing signal molecule inhibits lymphocyte proliferation and TNF-alpha production. By producing phospholipase C it furthermore suppresses neutrophils respiratory burst activity [117].

-Phagocytosis of *Staphylococcus aureus* and *Klebsiella pneumoniae* is inhibited by a polysaccharide capsule [103, 118].

-*Streptococcus pyogenes* produces a cysteine proteinase that liberates fibrinogen-binding and Fc-binding fragments of surface factors, thus liberating immune-complexes that become ineffective [119].

-Phagocytosed *Legionella* spp. are not killed because *Legionella* cytotoxin blocks neutrophil oxidative metabolism. *L. micdadei* produce a phosphatase which blocks superoxide anion production by stimulated neutrophils [120].

-Certain bacterial toxins aim specifically at immune cells, and are therefore classified here. Examples are *Staphylococcus aureus* alpha toxin [52] or *Actinobacillus pleuropneumoniae* hemolysin, which kills neutrophils and at sublytic dosis may impair

the oxidative metabolism of phagocytic cells [121]. The main target of *Bordetella bronchiseptica* adenylate cyclase toxin is phagocytic cells, so that these cannot eliminate the bacteria [34, 122]. Pore-forming toxins often use PMNs as targets before they can be killed by these cells [123].

• Opportunistic pathogens can make use of factors to evade immune responses. For instance, cytokine production is limited by an immunosuppressive factor from the opportunistic pathogens *Actinobacillus actinomycetemcomitans* [124] and *Porphyromonas gingivalis,* the latter produces proteinases that degrade the pro-inflammatory cytokine TNF-alpha and cleave a leukocyte C5a receptor so that chemotactic factors for PMNs are no longer formed [125, 126].

VII. *Regulatory genes of expression of virulence factors*

Expression of virulence factors is generally regulated at the transcriptional level [127, 128]. The regulatory genes will be detected as 'virulence' genes because their inactivation results in loss of phenotype. Since they are also involved in regulation of processes other than virulence they are considered virulence-associated genes here. Several common patterns of gene regulation can be recognized:

• Specialized sigma factors. Sigma factor RpoS (also known as sigmaS, KatF, Sigma 38) has been described as a general stress response regulator that controls the expression of genes which confer increased resistance to various stresses in some gram-negative bacteria. Many virulence factors involved in intracellular survival are under RpoS regulation [129-137]. RpoN, also known as Sigma 54, is usually involved in transcription of genes in response to environmental signals, including several virulence factors [133, 134]. Sigma 28 is a sigma factors specialized in flagellar biosynthesis [138]. Together with specialized sigma factors, transcription of virulence genes can furthermore be regulated by antisigma factors [139]. In the case of *Mycobacteria*, an attenuated mutant contained a specific mutation in the principal sigma factor gene [140].

• Phase variation by inversion of genes or their promoters is a mechanism of transcriptional on/off switching of virulence genes that is described for a number of genes and species [141-143]. Inactivation of the invertase involved in this process would result in a decrease of virulence because of loss of repertoire for antigenic variation.

• Another general regulatory process for virulence genes is by osmolarity (induction at high osmolarities), and temperature, mediated by the degree of DNA supercoiling. Transcription of genes under such regulation is often is sigmaS dependent, with H-NS and/or IHF to contribute to its fine tuning [144-152].

• Genomic integration of plasmid-coded virulence regulons can result in a change (either a decrease or an increase) of virulence. A model system is *Shigella flexneri* with similar regulons in *E.coli* and *Shigella* spp. [128].

• Global regulatory systems are described for a number of pathogens, and usually comprise of a sensor and an affector [153, 154]. Examples are:

-ferric uptake regulatory gene plays a crucial role in gene expression under iron-restriction, although this may not always be directly fur-mediated [155-157].

-Two component regulatory system *PhoP-PhoQ* in *Salmonella* regulates genes required for intracellular survival and resistance to cationic peptides [158, 159].

-In *Staphylococci,* autoinducing peptides activate a global regulator for the expression of genes encoding virulence factors and other exoproteins [160, 161].

- The gene *leuX*, which codes for a minor leucin tRNA and is associated with a pathogenicity island, acts as a global regulator by influencing the expression of various genes of pathogenic *E. coli* [162].

-*ToxR/ToxS* global regulators regulate transcription of virulence genes *in V. cholerae* [163, 164].

- Virulence of *Brucella suis* was demonstrated to be dependent of the global regulator system NtrBC [165].

• Specialized transcription activators are implied in regulation of a number of virulence genes. For instance, PrfA protein regulates the virulence genes of *Listeria monocytogenes*[157]. Transcription of the bundle-forming pili of enteropathogenic *E. coli* is regulated on a separate locus present on the EAF plasmid [166], and the *soxRS* regulon of *E. coli* coordinates the induction of at least twelve genes in response to superoxide or nitric oxide [167]. Transcription of the hemolysin operon of this organism is coregulated with pilus and LPS expression [168].

• As demonstrated for *Yersinia*, the type III secretion system plays a key role in coordinate expression of virulence factors after physical contact with the target cells [169].

• The gene coding for enterotoxin produced by *Clostridium perfringens* type A is absent in other types. Cloning of this gene Cpe into Cpe-negative strains results in correct expression, indicating that all other regulatory genes are present [170].

VIII. *Export, activation, and processing systems for virulence factors*

-In Gram-negative pathogens four distinct classes of secretion pathways have been identified that deliver virulence factors to their sites of action (surface-bound or secreted) [171-174]. In the type I secretion system, the secretion machinery is composed of three proteins forming a channel through the inner and outer membranes in a one-step mechanism. The secretion signal is present in the carboxy terminal region of the secreted protein but without proteolytic cleavage. In type II and type IV secretion systems, the crossing of the inner membrane involves the sec machinery with the cleavage of an amino terminal signal sequence. The crossing of the outer membrane involves the formation of a pore either by other proteins (type II) or by the carboxyterminal region of the protein (type IV). Type II secretion requires certain factors that are activated by specific cleavage by a protease [175]. Some genes with key functions in the type II secretory machinery, like the 'gatekeeper' protein, have backup versions, at least in *P. aeruginosa* [176]. The type III secretion system involves at least 20 proteins including cytoplasmic, inner membrane and outer membrane proteins which can inject secreted virulence factors into the cytosol of the host cells. Often protein secretion pathways are similar to those involved in assembly of bacterial appendages. Regulation of expression of secretory pathway components is used as a way of regulating virulence in *Bordetella* spp [177].

- The GroEL/HSP70 chaperonin of *Helicobacter pylori* is required for secretion of urease [68], which is an example of how chaperonins can affect virulence. Specific chaperonins are expressed by *Yersinia pestis* for Yop expression [178].

-regulation of by post-translational modification of virulence genes is often encoded by genes that are part of the same operon as the structural gene, e.g. the HlyA (hemolysin) of *E. coli* [179].

In conclusion, numerous examples can be found of virulence factors and virulence-associated factors for each proposed class. In the literature many 'virulence' genes (or, more carefully, 'putative virulence genes') are reported, that were identified by loss of virulence after inactivation. When examined more closely, many of these factors act as virulence-associated factors, or are involved in regulation of expression. It is important to consider the role of a single 'virulence' gene in the complete process of disease when the risk of genetic transfer of such genes should be assessed. Another important aspect of disease is the role of the host, which is treated in Chapter 3.

Chapter 3. The role of the host in virulence

The role of the host in virulence is as important for the outcome of disease as that of the infectious agent. Most clearly, the immune status of the host determines if colonization can occur after contact with infectious bacteria, and if so, in some instances the host influences the outcome of disease versus asymptomatic carriage. This is most apparent in immune-suppressed hosts, where otherwise commensal bacteria can become opportunistic pathogens. The effects of immunization by natural infection or vacciantion are not treated here. The immune response can limitate an infection to a certain location. The overall health-status of the host (as determined by nourishment, commensal microflora, other infections, organ failure, age) predestines the risk for complications. At the other extreme, some bacteria employ immune responses for their pathophysiology. A general pattern of the role of the host in bacterial virulence is therefore difficult to define, and host factors are probably as diverse as bacterial virulence factors are in determining bacterial virulence. In an attempt to incorporate the role of the host in the outcome of infection, the damaging effects of pathogenic bacteria can be given as a function of the host's immune status, not to be confused with the degree of humoral response due to prior infections (Figure 5, after [4]). Note that the different classes in Figure 5 represent classes of organisms, not genes, and that they are nominated with numbers, as opposed Roman numbers for the gene classes in Table 2. Further note that the curve of bacterial toxins would be completely horizontal, since these cause damage regardless of the function of the host's immune status. Examples of pathogens are given for each of these classes in the original publication. The prototypes of each class as proposed in [4] are:

- *Pneumocystis carinii*, which causes life-threatening pneumonia in patients with specific immunological deficits, particularly in those with AIDS, whereas individuals with a fully functional immune system are not affected by exposure to *P. carinii*, though antibodies are produced.
- *Streptococcus pneumoniae* is an example of a bacterium that can cause life-threatening pneumonia in normal hosts but more frequently in individuals at the extremes of age and with defects in humoral immunity.
- *Histoplasma capsulatum* is the prototype for microbes that can either infect asymptomatically, or result in severe damage, in this case pneumonia. Note that the distance

to the y-axis of the graph for class 3 organisms can be variable, as illustrated by the double curve.

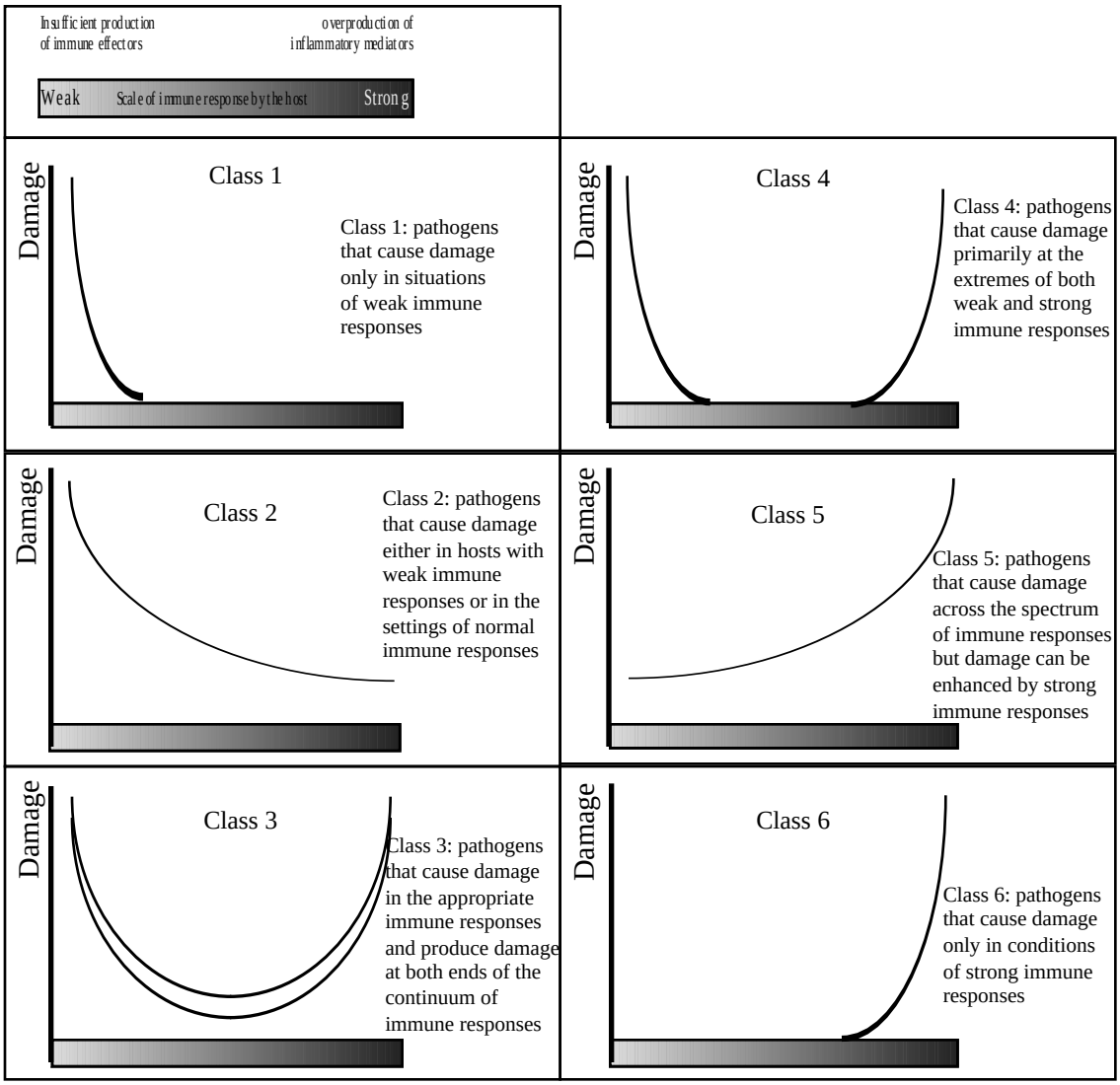
- *Aspergillus fumigatus* is the prototype of a class of microorganisms that cause symptomatic infections only in patients who have impaired immunity or protracted immune responses to the pathogen.
- *Campylobacter jejuni* is an example of a bacterium that can cause chronic damage due to excessive or inappropriate immune response. In this case, certain serotypes elicit a neurological auto-immune paralysis named Guillain-Barré Syndrome.
- *Helicobacter pylori* meets the criteria of a pathogen that is only pathogenic when the balance between organism and host becomes distorted, and a strong immune response results in damage, in this case in the form of chronic gastritis and peptic ulcers.

The numerous examples of pathogens classified in this manner that are listed in [4] illustrate clearly how important the influence of the host's immune response is on the pathology of an infectious disease. However, this is not the only host effect known. As in most biological processes, genetic predisposition is another factor of influence. For instance, certain Fc-gamma receptor polymorphisms result in increased virulence of pneumococcal and meningococcal infections [180]. In the case of Guillain-Barré Syndrome (GBS) as a result of a *Campylobacter jejuni* infection, unidentified host factors increase the risk of this post-infectious neurological auto immune disease, since only a small proportion of the infections with the GBS-related serotypes of *C. jejuni* actually result in neurological damage [181]. Host factors probably increase the risk of HUS after Shiga toxin producing *E. coli* infection [33]. The difference in susceptibility of mice to *Salmonella* infections is due to a polymorphism in the *ity/bcg/lsh* locus, which is involved in resistance to *Leishmania* and *Mycobacterium* infections as well. The Nramp1 gene of this locus encodes a membrane protein and a point mutation in this protein can limit the killing potential of macrophages for the mentioned pathogens [see 182 for relevant references].

A third way how host factors influence disease is by activation of bacterial virulence genes. This can be a specific, though unwanted, increase of virulence, for instance by activity of granulocyte colony-stimulating factor [183]; or, alternatively, unspecific

Figure 5.

Damage of pathogenic bacteria as a function of the immune response of the host (after [4]).



activation, for instance the effect that dietary changes have on the sterility of the mucosal lining epithelial of the intestines [184].

Host-specificity and tissue tropism are characteristic for infectious diseases. The host-factors determining these specificities are mostly receptors for primary colonization factors and are characterized in a minority of cases. The role of host-specific or tissue-

specific receptors in bacterial virulence is important in the context of this study, because they could be targets for altered virulence due to a host jump or a shift in virulence. Literature searches for publications on tissue-tropism of bacterial pathogens resulted in one example of such a shift in virulence: accumulated point mutations in the FimH lectin of type 1 fimbriae of *E. coli* are thought to have resulted in a uropathogenic strain originating from a commensal [185]. In general, the specificity of site of colonization can be seen as an integral part of the ecological niche to which the miroorganism is adapted, and therefore factors determining tissue-specific colonization are mostly covered in Chapter 2: *primary colonization factors* and *auxiliary colonization factors*. When closely related pathogens have different tissue tropism, comparative genetics can be used to identify the factors responsible for these differences. A comparison of *Neisseria meningitidis* and *Neisseria gonnorrhoea* revealed such potential genes [186]. When more genome sequences become available, this strategy will become more generally applicable.

Host-specificity requires an intricate interaction between host and microorganism. There is no fundamental rule that limits the number of host species that pathogenic bacteria can colonize. Nevertheless, all pathogens display a preference for certain hosts with varying degrees of specificity, ranging from different vertebrates, only mammals, to certain genera, or specific species. One way for a new pathogen to evolve is a change in host specificity by an existing pathogen (host-jump). The first step in this process is to gain the ability to colonize and cause disease in a new host, the second step is to allow the microorganism to be transferred between individuals of this new host, so that infection of this new host is not a dead-end. The first step can result in high virulence, which is reduced as the microorganism adapts to the new host (and vice versa). Host-jump as a mechanism for new virulent microbes to develop is well-known for viruses, with the HIV virus as a threatening example. Examples of bacterial pathogens are zoonotic infections, like *Campylobacter jejuni* and *E. coli* O157:H7, which are present asymptomatically in chickens and cattle, respectively, but cause disease in humans. It is not known at present why these organisms do not cause disease in the animal host. For *E. coli* O157:H7 the virulence factors causing disease in humans are well-characterized [33]. For *C. jejuni* this is not the case [187], despite the available complete genome sequence of one *C. jejuni*

strain [187b]. The lack of identification of virulence genes in this organism may be a reflection of our poor knowledge on both commensal and pathogenic host-specific interactions.

It is important to understand which bacterial factors are involved in host-specificity, because when these factors are shared between bacterial species they might allow a host jump to occur. Few examples of identified bacterial factors responsible for host-specificity (other than primary colonization factors), of for bacterial host jumps, were found. The following examples came from a literature search (1994-present) for host-specificity of bacterial pathogens.

-The host specificity of *Salmonella typhi* for humans, as opposed as *S. typhimurium* with a broad host-specificity (but which produces typhoid-like symptoms in mice), was used to perform subtractive hybridization between these two species. A beginning of the characterization of the genes specific for *S. typhimurium* has been made, however inactivation of at least one regulatory gene specific for this organism did not alter host-specificity [188].

-For *Listeria monocytogenes* the receptor of internalin InlA is E-cadherin. It was found that the binding site of E-cadherin for internalin is very specific for human (and not murine) E-cadherin, although the specificity of this site is not in the region involved in cell-cell interactions [189]. Although not described in this paper, it could be speculated that certain mutations in *L. monocytogenes* InlA could make it specific for murine E-cadherin, which would increase the virulence of this organisms in mice.

-*Pasteurella haemolytica* can colonize both sheep and cattle. By phylogenetic analysis of serotypes of isolates from both hosts, it could be determined that the ancestral host is the sheep, and that several distinct clonal lineages have crossed the species barrier to cattle, whereby certain OMPs are involved in host specificity and virulence [190].

-The virulence control system BgvAS of *Bordetella pertussis* and *B. bronchiseptica* are interchangeable [191]. The resulting chimeric organisms (*B. bronchiseptica* with a BgvAS locus of *B. pertussis*) showed no change in virulence in rats, but the sensitivity of the signal transduction system to factors influencing the phase transition (from virulent Bgv+ to avirulent Bgv- variants) had changed dramatically. These experiments show that virulence genes can be functional after exchange between related pathogens with

different pathogenicity, however their effect in a foreign genetic background can be different.
-The difference in host-specificity of *C. fetus* subspecies *venerealis* (infecting the genital tract of cattle only) compared to *C. fetus* subspecies *fetus* (which has a broad host-range and can cause enteric, genital, and systemic infections) is striking in view of their e genetic and phenotypic similarity. The host specificity and difference in pathogenicity is probably due to a minor genetic difference, probably restricted to one locus [192].

In conclusion, the role of the host in virulence cannot be ignored. As a consequence, bacterial genes are not solely responsible for the disease that is the outcome of an infection. The damaging effect that bacterial factors can have depends on the exposed site, the dose, the defense, and probably other variables. This complicates the demand for generalization, and also complicates the interpretation of the risk when virulence factors 'go astray'. In Chapter 4 examples are presented of virulence genes that are relatively recent additions to the genetic content of a given bacterial species. These examples can help identify likely strategies for future virulence to evolve, either spontaneously or by human activity.

Chapter 4. Evolution and spread of virulence genes

Three mechanisms can be proposed for the evolution of pathogens: (i) acquirement of virulence genes from existing pathogens by horizontal gene transfer; (ii) a change in host specificity (host jump) of an existing pathogen, possibly together with the acquirement of genes to adapt to a new ecological niche; and (iii) evolution of new virulence genes from the existing gene pool of a bacterial species, resulting in (an increase of) virulence. The evolutionary consequences of DNA uptake by horizontal gene transfer can be much more severe than the minute steps of mutation and selection of possibility (iii) [193]. Therefore the consequence of DNA uptake by horizontal gene transfer on virulence is further investigated; the accumulative effects of mutation and selection for the evolution of virulence are not considered here because they can not be measured or identified in general at present, and because these processes are less relevant in terms of experimental genetic modification.

An inventory was made of reported virulence gene transfer within and between species. The mechanisms of horizontal gene transfer are summarized. Examples were collected of virulence genes encoded on extra-chromosomal DNA, since these are likely candidates for horizontal transmission. Indicators for horizontal gene transfer of chromosomally encoded virulence genes are discussed. In a few cases the evolutionary origin of 'foreign' genes was discovered and examples of the evolution of certain virulent bacteria are summarized. Experimental horizontal gene transfer by genetic modification is compared and contrasted with natural horizontal gene transfer.

4.1. Evidence for recent acquirement of virulence genes

For a number of (virulence) genes it has been indicated that they were acquired by bacteria at a certain stage of their evolution by horizontal gene transfer. The terminology used in this chapter is explained in the box below. Some decades ago it was believed that virulence genes were exclusively present on episomes. Nowadays it is recognized that such extra-chromosomal, self-replicating DNA, for instance plasmids or bacteriophages, are likely, but not the only carriers of virulence genes, and that the location is not

constant for a given gene. Extra-chromosomal DNA is frequently transmitted between chromosome and episome, or between bacteria within or between species. Traditionally, bacteria were described to have a single circular chromosome with possible additional extra-chromosomal vectors, like plasmids or bacteriophages. All DNA taken together was considered the genome (although that term is now more generally used for the chromosome exclusively). Per definition the chromosome would be self replicating and contain essential genes, including ribosomal genes. The extra-chromosomal elements would contain non-essential, accessory genes. This view is no longer correct [194, 195, 205b]. Some bacteria contain more than one chromosome (for instance *Brucella* has two [194b]) or harbor plasmids the size of chromosomes, which can contain essential genes, including rRNA genes, as in *Borrelia* [195]. Bacterial chromosomes can be linear [196]. The size of a chromosome can vary considerably within a species (from 2.4 to 6.3 megabases in *Bacillus cereu*s), and even the number of chromosomes is not always constant, as it varies in *Brucella suis* strains [195, 197]. In view of horizontal gene transfer the distinction of chromosome and episome is irrelevant, since genes can migrate between these.

Genetic elements that can translocate are collectively called 'mobile elements' (a somewhat dated but accurate term) and are described in detail in section 4.2. Virulence genes present on mobile elements can be transferred between bacterial strains, subspecies, or, less common, between species. Plasmid-encoded virulence genes are common among bacteria and even when they are permanent components of plasmids, their transcription can be regulated by an intricate interplay of plasmid-encoded and chromosomally encoded transcription factors. Chromosomal integration of mobile elements can trap virulence genes that are normally encoded on the chromosome, and subsequently these genes can move with the element and eventually become integrated in a new chromosomal or plasmid environment. Virulence genes that are put in a new environment by mobile elements can be recognized because such elements often remain 'footprints' in the form of (incomplete) insertion sequences. Often clusters of genes are thus translocated, and when (some of) these are involved in virulence, these loci are named pathogenicity islands (PAI's). Since the inserted genes are often derived from genomes with a GC content different from the recipient organism, PAI's are characterized

Horizontal gene transfer: mobilization and stabilization of genetic information from organism to organism.

Gene flux: the pool of DNA that is available to and shared between cells, not necessarily belonging to the same species.

Genome plasticity: evidence for differences in the linear order of genes on the genomes among members of a given species.

Chromosome. A bacterial chromosome can be defined as a DNA molecule that contains the necessary information for replication and continued life of the cell under optimal growth conditions. Integrated plasmids and phages are regarded as temporary guests, crippled (immobilized) mobile elements as permanent residents.

Conjugation: transfer of DNA from donor to acceptor during cell-cell contact. This contact is generally enabled by pili (protein cylinders).

Natural transformation: the uptake and incorporation of exogenous DNA without cell-cell contact. This is restricted to certain bacterial species which are called naturally competent. Such species could bridge the distance between conjugation-incompatible groups of species.

Transduction: transfer of DNA by bacteriophages (viruses living in bacteria). During transfer the DNA is protected against degradation and DNA transfer is independent of competence, however specific phage receptors must be present.

by a GC-content deviating from the rest of the chromosome, and they are often inserted in tRNA genes. The original definition of a PAI indicated all these features (deviant GC content, flanked by insertion sequences or repeats, inserted in a tRNA gene, contains virulence genes) as characteristics of a PAI [198], however more recently any clustering of virulence genes have been (mis)named PAI, regardless of other characteristics that indicate they were recent additions to the bacterial chromosome. A deviant GC content,

or an atypical codon usage (codon bias), is often interpreted as an indicator for recent DNA transfer. It is argued that such genes may have been inserted by mobile elements or by transformation, and that they have not been present in their new environment long enough to evolve to the preferred codon usage and GC content of the recipient genome by accumulative mutation. This may or may not be correct, since it is not known whether all genes obey the same pressures of mutation. It is possible that a deviant GC content or atypical codon usage has evolved and is maintained for reasons of regulation of expression, and that virulence genes are more frequently regulated in this way than other genes. Although analysis of codon usage bias of completely sequenced genomes grouped out genes of prophage origin and genes with low translation efficiency [199] (in favor of both foreign origin and regulatory mechanisms), the question was not addressed whether a codon bias does predict a foreign origin, a low translation efficiency, or both. Moreover, deviation from the mean codon usage and GC content tends to increase with the distance from the origin of replication [200]. Therefore, a difference in base composition is not sufficient to assume a recent DNA transfer for genes. Supportive evidence is discussed in section 4.3. The next section deals with horizontal gene transfer by means of mobile elements.

4.2. Horizontal gene transfer by mobile elements

There are several mechanisms of horizontal gene transfer described for bacteria leading to genome plasticity. Recombinations within the chromosome can change the order of genes. Transfer of genetic material between strains or species can take place by conjugation, transformation or transfection. Mobile elements are of key importance for such gene transfer; they can be devided in transposons, integrons, invertases, conjugative plasmids, bacteriophages, conjugative transposons, pathogenicity islands, and intermediates thereof (Table 3). Horizontal gene transfer enables the spread of evolutionary successful genes. The result is a degree of homogenization of genetic material which departs from bacterial lineage and has a much greater evolutionary influence than accumulative point mutations [193].

Table 3. Mobile elements.

Name	Description	Specific features	Replication
Bacteriophages	bacterial viruses: protein-packed DNA or RNA	transfection is receptor dependent	replication after integration or as episome
Conjugative plasmids	tra/mob-dependent conjugation	'trapped' DNA is trans-ferred by homologous recombination	OriT dependent self-replication
Conjugative transposons	combines features of transposons, plasmids, and bacteriophages		depends on integration
Transposon	flanked by inverted repeats and often by duplication of target sequences	contains its own transposase	depends on integration
Invertron	linear plasmid with inverted repeats at both ends	covalently bound proteins at 5'-end	depends on integration in chromosome, phage, or plasmid
Integron	part of transposon, also called 'gene cassette', often contains antibiotic resistance gene	inverted repeats are absent	depends on integration, recipient strain must have the integrase

The nomenclature of mobile elements is confusing and not always consistent. The general terms mobile elements or transposable elements are used for any fragment of DNA that can translocate within a genome or between genomes. Some mobile elements are made up of modules of individual elements, e.g. insertion sequences can be part of transposons, or invertrons can integrate in a phage or plasmid. The nomenclature has changed with time which makes it difficult to give definitions. In addition to table 3 these descriptions are compiled from the literature [193, 197, 201-206]:

Bacteriophages. These are often restricted in their infectivity but they can be very abundant in the environment, e.g. in seawater. Phages often carry virulence factors, e.g. toxins [202].

Conjugative plasmids are, besides phages, the most sophisticated modules for horizontal gene transfer. Conjugation is dependent on tra genes which are divided in three operons present on the plasmid. In addition the mob gene is required, and the conjugated DNA must contain OriT. After cell-cell contact (via pili) replication of the plasmid is activated. Factors are present on the cell surface to monitor incompatibility of plasmids. The transfer occurs via the 'rolling-circle mechanism' in which nicked single strand DNA is passed. The transfer machinery can act in trans, so that other DNA can also be transferred, as long as it contains OriT sequences. For conjugated DNA other than the conjugative plasmid, homologous recombination is required for survival, which means that sufficient homology must be present. Some conjugative plasmids are very promiscuous and can cross species borders. Others are restricted, e.g. by the lack of defenses against restriction enzymes. Conjugation was demonstrated between Gram negative bacteria, between Gram positives, between these two, between *E. coli* and yeast, and between *Agrobacteria* and plants (T DNA elements).

mobilizable plasmids contain an oriT and other mob genes but require a conjugative plasmid to be mobilized. Prototype is RSF1010 [201].

Conjugative transposons combine features of transposons, plasmids and bacteriophages [206]. They are normally integrated in the bacterial chromosome. After excision a covalently closed circular intermediate is formed (like a plasmid) but unlike a plasmid this cannot replicate. Instead it can integrate in a new location or be transferred by conjugation to a new acceptor cell. Their mechanism of excision and integration differs from that of transposons like Tn5 or Tn10 and resembles that of phages, however the DNA is not packed in viral particles. Unfortunately they are sometimes named Tn, like transposons. Conjugative transposons can mobilize co-resident plasmids and even, in the case of *Bacteroides*, unlinked integrated DNA segments. They are known traffickers of antibiotic resistance genes. Prototype: Tn916.

Transposons are mobile elements that are bordered by inverted repeats and flanked by duplication of target sequences, due to their mechanism of integration. A transposon always contains its own transposase gene [203].

invertrons are linear plasmids that contain inverted repeats at both ends, and are covalently bound to proteins at the 5' end [205]. They are found in bacteria, yeast, drosophila, maize, adenoviruses, and transposons. Invertases do not encode their own replication machinery and are integrated in replicons (viruses, phages, chromosomes, plasmids) for most of their lives. The prototype is adenovirus.

Insertion sequences (ISs) can exist as such, or flank genes that are 'trapped' by ISs. Most transposons depend on insertion sequences for integration, however class II transposons do not contain ISs but carry inverted repeats. Insertion of ISs is site-independent. Transposition of ISs has been proven in dormant bacterial cultures, that is in the absence of bacterial growth [205b].

Integrons are parts of transposons that can recombine within the transposon at hotspots. Transposons are recognized to be built up from modules that possibly arose from recombinations between integrons. A modern term for such modules is

Gene cassette, which comprises of a single gene (often an antibiotic resistance gene) and a specific recombination site within a larger mobile element [204]. A gene cassette can only integrate by site-specific recombination and is dependent on integrase encoded by the recipient integron. They do not normally contain a promoter nor are they flanked by inverted repeats. Gene cassettes often occur in pairs or groups.

Intermediates. ISs can integrate in phages or plasmids. Different mobile elements can integrate together in the chromosome. Phages can integrate in plasmids and become immobilized by loss of essential genes. Thus, there is overlap of sequences between most of these groups.

Retroviral integrases, also called retrons, also called Ty-like Transposons, show similarity to certain retroviruses and are not found in bacteria (only in eukaryotes). They contain direct repeats on both ends (not inverted repeats like invertrons) and carry their own replication genes, including reversed transcriptase since they replicate via RNA. Despite this difference in replication, the mechanism of integration is identical to that of transposases.

Mobile elements are primarily identified on the basis of sequence homology. Mobile elements can affect gene expression in a number of ways. Obviously the acquisition of genes will affect gene expression with possible consequences on phenotype, virulence, antibiotic resistance, etc. Many mobile elements carry genes involved in virulence. Apart from the addition of genes to the existing genetic content, insertion of an integron or transposon can also activate otherwise silent (promoterless) genes that are already present, e.g. by correct positioning of a promoter sequence. Alternatively silent genes carried by mobile elements can become part of an expression locus, whereas inactivation of formerly expressed genes by insertion of the mobile element (either by disrupting the open reading frame or by dislocating the promoter from its gene) is also possible. Finally, the expression of a gene is dependent on the DNA topology (e.g. supercoiling affects promoter activity) so that repositioning of a gene can affect its expression.
Common integration sites for bacteriophages, plasmids and PAI's (see below) are tRNA genes [202, 207], because they are highly conserved and often present in multiple copies, which are perfect properties for docking sites.

4.3. Horizontal gene transfer of virulence genes

Horizontal gene transfer of virulence genes has been demonstrated by plasmids, bacteriophages, insertion sequences, natural transformation, and by transfer mechanisms that could no longer be identified. Examples from the literature are given for each transfer mechanism.

Virulence genes encoded on bacteriophages

-The cholera toxin of *Vibrio cholera*e is encoded on a bacteriophage (CTXphi) [208]. In addition, a second bacteriophage (VPIphi, formally identified as pathogenicity island Vpi) encodes a toxin-coregulated pilus (TCP) that is the receptor for CTXphi. Thus, both phages are required for toxin production (and for virulence), and one phage encodes the receptor for the other [208]. In the laboratory the enterotoxin genes of CTXphi can also transfect into *V. cholerae* strains that lack the receptor for CTXphi (TCP), using an alternative phage. The existence of CTXPhi positive strains that lack the TCP receptor suggest that this alternative route also occurs outside the laboratory [209].

-The shiga toxins 1 and 2 of *E. coli* O157:H7 (causative of hemorrhagic colitis and hemolytic-uremic syndrome) are phage encoded [210].
-Several other bacterial toxins are encoded on a bacteriophage as summarized in [202]: diphteria toxin produced by *Corynebacterium diphteriae*, *Streptococcus pyogenes* toxin, *Staphylococcus aureus* enterotoxin A, *Clostridium botulinum* neurotoxin, and the cytotoxin and pili of *Pseudomonas aeruginosa*.
-Capsule production by *Streptococcus pneumoniae* is phage-encoded [211].
-Virulence associated genes in *Dichelobacter nodosus* (causing footrot in sheep) are flanked by an integrase gene adjacent to a tRNA gene, indicative of an integrated bacteriophage [202, 212]. The virulence-associated open reading frames are closely related to plasmid-borne genes of *N. gonorrhoeae*, *E. coli*, and other bacteria, and this gene organization fits all requirements of a pathogenicity island (see below).
-Bacteriophage-transmitted DNA transfer was suggested as a general mechanism for evolution and transfer of bacterial virulence determinants and as a major transmission mechanism for pathogenicity islands [202, 211, 213].
-A cryptic bacteriophage is found in highly virulent *Salmonella typhimuruim* strains that carries a superoxide dismutase gene different from the classical SOD gene (which is homologous to the gene found in *E. coli*) [78]. When the prophage is cured, virulence is decreased [214]. A second prophage is identified that can partly overcome attenuation when the first phage is cured. The virulence genes on this second phage have not yet been identified [208].

Virulence genes encoded on plasmids
-Many plasmids are identified in *E. coli* strains, some of which harbor virulence genes, especially genes coding for fimbriae, intimin, and toxins [33, 215, 216]. Chromosomally-encoded virulence genes can be regulated by genes on plasmids (see for example [80, 215, 217]). Virulence genes encoded on plasmids can also be regulated by chromosomal genes, e.g. by RpoS in *Salmonella* spp [218, 219]. Regulation of virulence genes on plasmids can be very complex and sensitive to extracellular conditions [144-149]. The completely sequenced plasmid of *E. coli* O157:H7 will be discussed below.

-A 70 kb virulence plasmid of *Yersinia* spp. encodes the Yop virulon (virulence operon) which encodes genes needed for adherence and for secretion and injection of bacterial effector proteins (Yops) into eukaryotic cells to disable them. The plasmid also encodes a type III secretion apparatus. The Yop proteins disrupt cellular communication or induce apoptosis of immune cells. Transcription is temperature regulated and all regulatory genes are included on the plasmid [110, 111, 178, 220].
-A virulence plasmid of *Shigella flexneri* encodes invasion genes which are temperature-regulated by a regulon also present on the plasmid, and which are silenced when the plasmid is cured or integrated in the chromosome [148, 150].
-*Bacillus anthracis* contains virulence genes on two plasmids, which can be used for positive genetic identification by PCR. Capsule formation is encoded by one plasmid and is regulated by a trans-activator encoded by the other [221, 222].
-Various individual virulence-associated genes have been located on plasmids. One example is a virulence-related plasmid of *Rhodococcus equi* that is responsible for intracellular growth [223, 224]. Recently a urease gene of *Clostridium difficile* was identified on plasmid, although a function in virulence has not been established [225].
-In *Borrelia burgdorfer*i a linear plasmid was found to contain telomeric fragments on its ends, similar to fragments present on African swine virus. This virus is carried over by the same tick vector. The telomeric sequences are possibly of human origin [226].

Virulence genes associated with insertion sequences
-A site-specific insertion sequence IS1301 in *Neisseria meningitidis* results in inactivation of a gene regulating capsule expression. Encapsulated *N. meningitidis* cannot adhere and invade epithelial cells and are thus avirulent [227]. This example of regulation of virulence expression by insertion sequences illustrates how such small insertion sequences can have major consequences on virulence.
-Two catalase-peroxidase genes were identified in *Mycobacterium fortuitum* (an opportunistic pathogen), one of which in the vicinity of IS6100. The two genes were less related to each other than to catalase genes of other organisms, which suggests that they did not originate from gene-duplication but from gene acquisition [228].

Virulence genes associated with other mobile elements

The mobile elements of the other classes are dependent on chromosomal integration for replication (Table 3). Thus, virulence genes introduced by such elements are most likely found on the chromosome. Such virulence genes are nowadays mostly identified as part of a pathogenicity island, and their origin of transfer is not established, either because the mobile elements are no longer (completely) present or because the differentiation of the various mobile elements is no longer fashionable. Even when the vehicle of transfer is identified, the term pathogenicity island is used, e.g. in the case of a bacteriophage that was identified as the source of a PAI in *Vibrio cholerae* [208]. Examples of well-characterized pathogenicity islands are given below. Transposons are frequently involved in intra-molecular transitions (relocation of a DNA fragment within a chromosome). Through their action they can cluster virulence genes together and may have helped in the formation of pathogenicity islands [201]. The association of integrons with virulence genes is discussed in section 4.5.

Pathogenicity islands on chromosomes

Virulence gene loci that are thought to be recent additions of the chromosome are nowadays known as pathogenicity islands. The insertion site of PAI's is often a tRNA gene which is implicated in the recombination process [207]. The gene cluster encoding a type III secretion system has become synonym to a pathogenicity island, however PAI's do not always contain these secretory genes. Below follows a collection of examples of clustered virulence genes for which evidence is available that they were acquired by horizontal gene transfer.

-In *Salmonella enterica* 5 PAI's have been recognized, called SPI 1(for *Salmonella* Pathogenicity Island) to SPI 5 [229-234]. SPI 1, positioned at 63‘, renders *Salmonella* the ability to invade epithelial cells and encodes a type III secretion machinery [213]. SPI 2, at position 31‘, mediates survival within macrophages and bears a second copy of a type III secretion locus. SPI 3 was identified by its position on the chromosome: it is inserted in *selC*, a locus in which a PAI is present in several *E. coli* strains. SPI 3 has a mosaic structure and can vary between serovars, and a mosaic nature was also reported for a PAI encoding an iron uptake system [232].

-In *E. coli* several PAI's are now recognized that are specific to the pathotype [33, 213, 235, 236]. PAI-1 is found in uropathogenic *E. coli* and is integrated at the same site, selenocysteine tRNA, as LEE (for locus of enterocyte effacement, on which the genes for attaching and effacing are located) which is present in enteropathogenic *E. coli*. In addition at least 3 other PAI's have been identified, several of which contain hemolysin genes. Multiple islands can be inserted in one genome in different tRNA's. For two PAI's the activity of a phage is suggested as the mechanism of transfer [213].
-In *Yersinia* spp. a PAI is recognized that is flanked by direct repeats, contains IS100 sequences, and encodes a putative integrase, which are probably remnants of the transfer vehicle. The PAI contains the iron uptake virulence genes and is inserted in different tRNA genes in each *Yersinia* species, suggesting horizontal transfer after speciation [237].
-In *Helicobacter pylori* a pathogenicity island carries the virulence genes *cag*A and *vac*A. The PAI is a hotspot for mutations and deletions and is (partly) absent in avirulent strains, so that it is used as a marker to identify virulent forms of this gastric bacterium [238].
-*Clostridium perfringens* toxin genes are located at a pathogenicity locus (PaLoc) which is absent in less virulent strains. The locus itself does not have the characteristics of a mobile element. More likely it is part of a larger element which would nowadays be called a PAI [239].

Pathogenicity islands on virulence plasmids

Pathogenicity islands can be part of virulence plasmids. The complete DNA sequence of the 92 kb plasmid of *E. coli* O157:H7 demonstrates how virulence plasmids can be mosaic of structure, with insertion sequences, pathogenicity islands, and other genes most probably acquired by horizontal gene transfer [240]. The sequence has revealed a number of newly identified open reading frames, including a large ORF with significant homology to the active domain of *Clostridium* toxin *toxA*. This domain encodes the glycosyl transferase activity in *Clostridium* spp. with specificity for G-proteins. The other domain of ToxA, responsible for trafficking the active domain into the target cell, is missing on the *E. coli* plasmid. Although the type of disease observed with *E. coli* O157:H7 is similar to that of *Clostridium difficile*, a role of the large plasmid in virulence has not been clearly established, and the observed gene similarity between the ORF and

ToxA has yet to be confirmed by functional analysis. Further characteristics of the plasmid suggest that it is formed by a collection of genes and loci from different origins, as suggested by segments with different codon bias and GC content, which are separated by complete or incomplete insertion sequences [240]. Interestingly a type II secretory pathway is present on the plasmid, and probably also on the chromosome. Phylogenetic analysis suggests that the locus on the plasmid originated from a different species and not from the chromosomal ortholog [240]. Thus, the mosaic structure of the plasmid resembles and magnifies the mosaic nature of pathogenic loci on chromosomes.

Horizontal gene transfer by natural transformation

The natural ability of many bacterial species to take up DNA (natural competence) is a major route for horizontal spread of genes, be it on mobile elements or on chromosomal DNA. The importance of natural competence in bacterial evolution has become evident [240b]. The mechanism of natural transformation of both Gram-positive and Gram-negative bacteria is largely understood [240c].

Evidence for horizontal spread of virulence genes by natural transformation without the activity of transfer vehicles would be: an observed difference in GC content, with supportive evidence such as phylogenetic analysis that results in a tree different from conventional expectations [200, 241]. There are some clear examples of prokaryotic genes found in a eukaryote (e.g. Fe-superoxide dismutase in *Entamoeba histolytica*), or the other way round (e.g. glucose-6-phosphate isomerase in *E. coli*) [241] (note that these examples do not describe virulence genes). It has been observed that informational genes (encoding for factors involved in replication, transcription, translation, and related processes) are less likely to be horizontally tansferred than operational genes that participate in housekeeping or auxiliary functions, including virulence [242]. When the genomes of two spirochetes were compared (*Treponema pallidum* and *Borrelia burgdorferi*) most orthologs (genes in different species that are related by vertical descent, i.e. have a common ancestral gene) were found in genes involved in 'core functions' (replication, repair, transcription, translatation, cell division) [243]. An explanation for this observation is given by the complexity hypothesis, where it is assumed that informational gene products are involved in such complex processes (which

may involve up to 100 proteins) that this decreases the chance for a successful transfer [241]. If this theory is correct, virulence genes are also expected be more prone to transfer than informational genes. However, even informational genes can be horizontally tranferred, as demonstrated by the topoisomerase I gene of *B. burgdorferi,* which did not have an ortholog in *T. pallidum,* and which grouped with *Cyanobacteria* in a phylogenetic analysis [243]. Such cases indicate that transfers can occur between species that are genetically distantly related. For these intracellular pathogenic spirochetes, this must have occurred in the past when their ancesters still lived in close association with other bacteria. The time of transfer cannot be determined accurately, though it can be established whether it occurred before, or after the two spirochetes parted. Analysis of these two genomes indicated the major role of horizontal gene acquisition in the evolution of the specific lifestyle of these two pathogens [243]. The frequency of exchange of operational genes can differ for reasons not yet understood. Phylogenetic analysis of the adenylate kinase gene and the RecA gene from different *Neisseria* species revealed major differences in DNA transfer of these two genes between the species [244]. This paper also describes computational tests that can identify transfers of partial genes, for instance the index of association between codons and the Sawyers runs test [244]. Examples of virulence gene transfers between pro- and eukaryotes were not found in the literature, however transfers from eukaryotes to prokaryotes of genes other than virulence genes are described (for example in [241]). Genome analysis of 21 bacterial and eubacterial genomes has revealed that the intracellular parasites *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Borrelia burgdorferi* and *Treponema pallidum* have a significantly higher number of eukaryotic genes than other pathogens or non-pathogens [245], however these genes are mostly not recognized virulence genes, and whether the observed difference is a reflection of intracellular lifestyle remains to be demonstrated. Phylogenetic evidence for horizontal gene transfer of virulence genes by transformation between prokaryotes was obtained for capsule genes of *E. coli* with a high similarity to *Streptococci*. The evidence was enforced by analysis of GC content, codon bias, and dinucleotide frequencies [246]. In one case strong evidence for natrual transformation was provided: the SodC gene in *Neisseria meningitidis* was found to be flanked by uptake sequences of *Haemophilus influenzae* [247]. Both organisms are naturally

competent but their uptake sequences differ. Nevertheless DNA seems to be able to cross this species border [247]. Only weak evidence, based on GC content, suggested that LPS genes of *Brucella* were acquired by horizontal gene transfer [248]. The pathogenicity locus of *Clostridium difficile* was identified as a specific insertion of 19 kb to replace 115 bp present in non-toxinogenic strains [239] however the mechanism of tranfer of this locus was not identified. The difficulty of identifying horizontally transmitted genes may eaven be greater in case genes are not transmitted completely. When relevant segments of genes are transmitted and fixed by selection rather than complete genes it becomes extremely difficult to identify such events, although their effects may alter virulence just as much as the transfer of complete genes [249]. Methods have been developed to identify 'xenologs', genes acquired from horizontal gene transfer, from complete bacterial genome sequences [248b, 248c].

Classes of virulence genes that are most likely to be transferred

The virulence genes that were proved candidates for horizontal gene transfer can be sorted according to the classification suggested in Section 1.6. This is illustrated in Table 4.

From Table 4 it can be seen that primary virulence factors (toxins and invasins) and fimbriae are often encoded on plasmids and phages, as has been recognized in the past, and that these are the most likely candidates for horizontal gene transfer of virulence genes. When more information on the genetic ancestry of virulence genes becomes available, e.g. by population genetics and genome sequencing projects, it may be possible to predict which other classes (if any) of virulence genes have a significantly higher chance for horizontal gene transfer. At the moment the available data are not sufficient to recognize such a trend. The possibilities and consequences of gene flux among pathogenic bacteria are discussed in the next section.

4.4. Evolution of virulence by horizontal gene transfer

The importance of horizontal gene transfer (hgt) for the evolution of pathogenic bacteria is suggested by the findings that many virulence genes are present on pathogenicity islands and on (or associated with) mobile elements, as illustrated in Section 4.3. The

Table 4. Horizontal gene transfer of virulence genes

Virulence gene class	Description	Vehicle of transfer	Organism	Reference
Primary vir.factor	Shiga toxin	phage	*E. coli*	210
	Cholera toxin	phage	*V. cholerae*	208
	Anthrax toxin	plasmid	*B. anthracis*	221, 222
	Enterotoxin	plasmid	*E. coli*	256
	Invasins	plasmid	*Yersiniae*	
	Invasins	PAI	*Salmonellae*	
Primary col. factor	Fimbriae, adhesins	plasmid	*E. coli*	256, 216
Auxiliary col. fact.	Iron uptake	PAI		232
	Superoxide dismutase	Trans-formation	*Neisseria meningitidis*	247
	Capsule	Trans-formation?	*E. coli*	246
Immune system evasion	Superoxide dismutase	phage	*Salmonella*	78
	Yops	plasmid	*Yersiniae*	111
regulation	Diverse	plasmid	diverse	

effect of hgt in the evolution of a pathogenic bacterium is described for a few pathogens. For *Salmonella enterica* and its closely related *E. coli* it is more likely that their common ancester was a commensal, and that subsequent addition of virulence genes made *Salmonella* pathogenic (as opposed to the loss of virulence in *E. coli* from a pathogenic ancester) [251]. Population genetic analysis by multilocus sequence typing (MLST) has revealed the most likely evolution of *Yersinia pestis* from *Yersinia pseudotuberculosis* by acquirement of a virulence plasmid and chromosomal virulence loci [252]. In this case it could even be indicated at which time scale these events took place. To find such strong

and direct evidence of hgt in the evolution of bacterial pathogenicity is very laborious and will not be easy for pathogens where the primary virulence genes are not identified. Note, however, that population genetic analysis does not use virulence genes as markers directly, because these are under stronger selection pressure than housekeeping genes.
One can only speculate about the age of PAI's as long as there are no supportive phylogenetic data available. In one case evidence obtained from *E. coli* suggests that the ancesters of certain PAI's were obtained after speciation, and it is speculated that they may have had a role in the development of pathotypes [253]. For *Salmonella enterica,* the pathogenicity island SPI 1 was already present in the last common ancester of all comtemporary lineages of *salmonellae* [250]. This excludes the possibility that *Salmonella* received this pathogenicity island from *Shigella flexneri,* since the *inv/spa* locus (with the type III secretory genes, known in *Shigella* as the *mxi/spa* locus) of the latter organism is a more recent addition. Transfer of this locus from *Salmonella* directly to *Shigella flexneri* can also be excluded because the GC content of the *Shigella* PAI is different from that of *Salmonella* [251]. The second type III secretory locus present on SPI 2 of *Salmonella enterica* is a later addition than SPI 1, since it is absent in the most divergent subspecific group of *S. enterica*. The addition of this second PAI was probably a key step in the evolution of intracellular survival of this pathogen [251].
It may be difficult to specify when a genetic transfer took place, but it is even harder to identify the source of the incoming DNA. In the case of the PAI present on the *E. coli* O157:H7 plasmid it is suggestive to indicate a *Clostridium* species as the source of the putative *toxA*-like cytotoxin gene, based on the observed homology. However, such analyses are completely dependent on the input of available sequence data, and an alternative 'donor' species with stronger homology may be identified when additional sequences become available. For that reason a source of transferred DNA is normally not identified. The PAI in which the *toxA* gene is present has a GC ratio different from the *E. coli* chromosome, however the original publication describes this ratio as 'typical for a bacteriophage' and does not compare it to that of *Clostridium* [240].
The appearance of *E. coli* O157:H7 in the foodchain in the early '80s raised the question whether this strain had newly evolved, or improved detection methods had resulted in its identification. Analysis of historical culture collections revealed that the strain had been

around before, but that it has since become acid resistant so that it can survive in food. All isolates of this strain represent a clone, with a striking virulence marker, shiga toxin (Stx), present on a lambdoid phage. Stx genes are also present in *Shigella dysenteriae*, where they are chromosomal. The evolution of *E. coli* O157:H7 is thought to be stepwise, from an ancestral EPEC-like strain, in which the uptake of the LEE pathogenicity island, the *stx* phage encoding shiga toxin, and of the large virulence plasmid were the key events. The origin of these transferred genes cannot be determined at present. However the lambdoid *stx* phages are also found in *Citrobacter fruedii* and *Enterobacter cloacae* strains, indicating that the *stx* genes can spread among different bacterial species [33].

Insertion of a PAI is an important step in the transformation of a benign organism to a pathogen. However, the genes encoded on a PAI must match with the regulatory sequences present on the acceptor chromosome (not all regulons are present on every PAI), and the additional genes must have a function in the lifestyle of the organism [193]. To illustrate such additional requirements for virulence phenotype, a 10 kb region was identified in the pathogenic species of *Listeria* (*L. monocytogenes* and *L. ivanovii*) that is also present in the chromosome of the nonpathogenic *L. seeligeri* [254]. The difference is thought to result from down-regulation of the virulence genes in the avirulent organism. If such 'virulence silencers' were deleted this would result in an increased virulence [255]. Such an event in the evolution of virulence genes is difficult to identify, but could in theory result from the activity of mobile elements. Repressors of inflammatory mediators, such as YopJ in *Yersinia pseudotuberculosis*, are postulated as silencers of inflammation for commensal bacteria [110], and deletion of such silencers could, at least in theory, induce a shift from commensal to an (opportunistic) pathogen.

Acquirement of a virulence gene may not be sufficient for (increased) virulence when the introduced gene is not properly transcribed, when transcription is not regulated, or when the produced protein is not properly secreted. In this respect it is noteworthy to mention that enterotoxin genes (CPE) are only present in 5% of *Clostridium perfringens* isolates. Nevertheless, strains that are CPE negative can regulate transcription of an artificially introduced CPE gene, which suggests that the regulation genes are already present, presumably because they are also needed for other processes [170].

Acquirement of a gene that is not coding for a virulence factor but for an antigenic determinant can have a major effect on virulence. This is illustrated by *Vibrio cholerae*. The pandemics of *V. cholerae* O:1 resulted in acquired immunity in exposed areas, however in the '90s a new epidemic in Bangladesh was caused by serotype O:139. The virulence genes (*ctx*) of this newly evolved serotype of *V. cholerae* were unchanged, and it is most likely that a clone of O:1 has changed its serotype to O:139 [256]. The combination of a new antigenic variant with the existing virulence genes resulted in lack of herd immunity and a new epidemic was born, in which the new serotype initially completely replaced O:1. A putative IS sequence was identified that was possibly involved in this gene acquisition; interestingly similar IS sequences were detected in other *Enterobacteriacae* [256].

For certain infectious diseases it is difficult to establish the role of individual virulence factors, due to the diversity of strains found to cause such infections. An example is extra-intestinal *E. coli* infections. In an alternative approach to identify virulent lineages of strains causing these infections, phylogenetic data were compared with lethality in a mouse colonization model [256a]. The result was, that a divergent lineage of isolates were the most virulent, and also contained most characterized virulent factors (as compared to *E. coli* strains from intestinal infections and commensal isolates). The authors assume that the remaining genetic background of this lineage must provide an advantage to virulence, however the strong correlation between virulence and phylogeny may also indicate that this lineage is a relatively young branch, with the acquisition of virulence genes at it's branch point, and that time has not allowed for further divergence [257]. With the growth of genomic and genetic databases, analyses like these can help identify evolutionary steps in the process of virulence development.

4.5. Natural barriers and selection pressures of horizontal gene transfer

In most cases plasmids are only transferred between strains within a species. This species barrier prevents rapid spread of virulence genes (as discussed in [257]), however spread can occur between species at a lower rate. Two species that are phylogenetically closely related and occupy similar niches are more likely to share virulence genes, e.g.

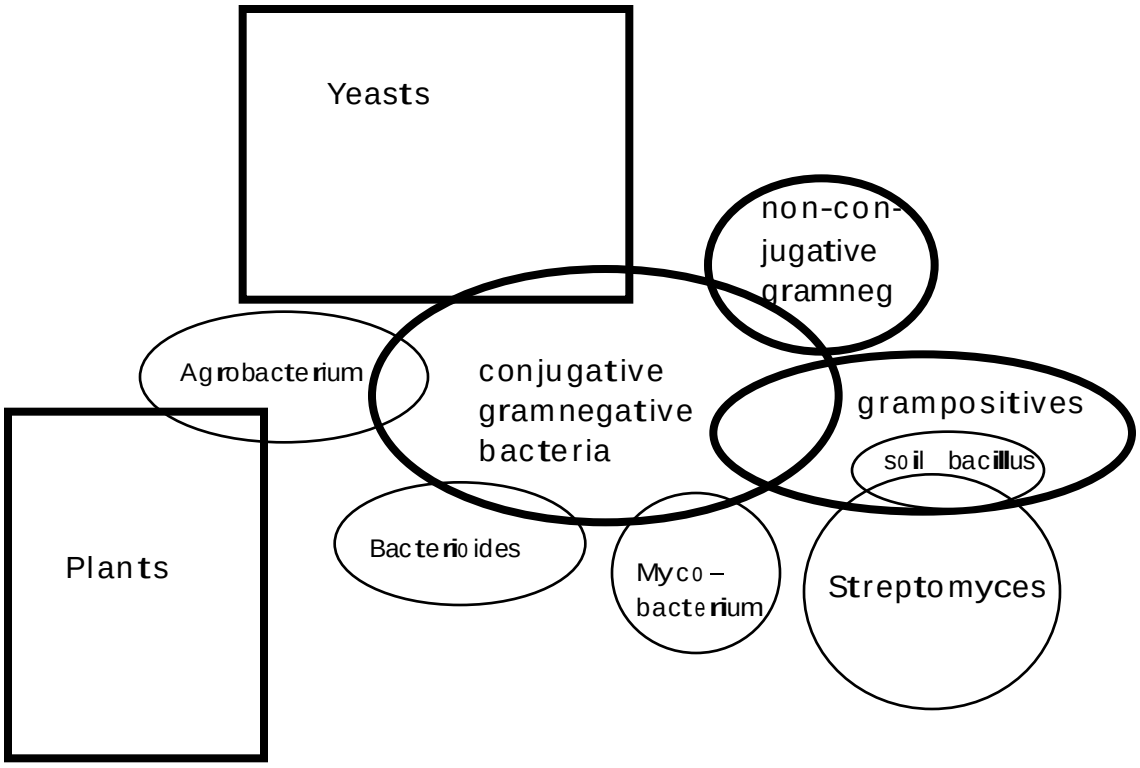
Salmonellae and pathogenic *E. coli*. Still, phylogenetic analysis of enteric pathogens that utilize a remarkably similar invasion machinery (like *Shigella* and *Salmonella* spp.) suggests that these mechanisms developed independently in these divergent pathogens [258].

Horizontal gene transfer is regulated by environmental factors. It has long been recognized (though sometimes ignored) that hgt between pathogenic organisms can take place in the host at a frequency higher than observed under laboratory conditions [259]. The host factors that regulate DNA transfer in vivo have not been identified in detail. It has been suggested that host-factors induce transfection of the bacteriophage carrying cholera toxin in *Vibrio cholerae* [259].

The selection pressures that select for carriage of virulence genes can not be separated from the pressures that select survival of the organism in the specific niche. However, many mobile elements bear antibiotic resistance genes as well as virulence genes. The spread of antibiotic resistance genes is not subject of this treatise, but it seems relevant to address the topic in a separate study. In modern practice of human and veterinary medicine there is a strong selection for spread and maintenance of antibiotic resistance genes. The observed clustering of antibiotic resistance genes within the gene flux [260] could be a model for clustering of virulence genes in PAIs. Although it is not a surprising finding that hgt of antibiotic resistance genes already existed before the introduction of antibiotics in modern medicine [260], present-day selection pressures have greatly increased the spread of such genes, and this selection pressure may indirectly aid the spread of virulence genes.

Horizontal gene transfer can take place between apathogenic and pathogenic bacteria. For instance, bacteriophages specific for *Streptococcus thermophilus* (an apathogenic bacterium used in milk fermentation) contain genes with homology to *E. coli* phages and *Mycobacterium* phages [261]. Although in this case virulence genes are not involved, it can be speculated that apathogenic bacteria can serve as an intermediate to transfer genes between species that are genetically incompatible. For instance, *Enterococci* are 'professional collectors' of antibiotic resistance genes, and can cause nosocomial infections in immune-repressed hosts [262]. They are abundant in the intestinal tract and can be present in food, accidentally or on purpose [263]. Although the frequency of their

Figure 6. Range of plasmid-related gene flux (after [201]).



transformation under natural conditions (in sewage sludge) was found several magnitudes lower than under laboratory conditions, gene transfer taking place in the municipal sewage water treatment plant of the city of Regensburg was estimated ranging from 10^5 to 10^6 events per 4 h [263b]. In contrast, *Enterococci* can be transformed under natural conditions in vivo at a rate higher than found under laboratory conditions. With genetically modified *Enterococci* in the avian gut the maximum in vivo transfer rate of a plasmid was found 0.03 transconjugant per recipient cell, a frequency that could only be obtained under laboratory conditions with forced filter mating [263c]. Thus, *Enterococci* can take up and pass on DNA under natural conditions, and theoretically serve as an intermediate between bacterial species that cannot directly share their gene pool.
Figure 6 illustrates the gene flux of plasmid related genes between bacterial species (from [201]). In this illustration all plasmid-encoded genes are included (most of which are not involved in virulence) but the figure nevertheless illustrates how certain species and

vehicles can bridge incompatible transfers. Furthermore the figure illustrates that there is no principal barrier for hgt between Gram-positive and Gram-negative bacteria.
A recent publication stated that "horizontal gene transfer is widely recognized as the mechanism responsible for the widespread distribution of antibiotic resistance genes, gene clusters encoding biodegradative pathways and pathogenicity determinants". It was even proposed that hgt "is also responsible for speciation and sub-speciation in bacteria" [263d]. Nature is our biggest experiment. It can be assumed that all mechanisms that allow bacteria to share the gene pool of different species, subspecies, and strains, have been explored and applied by Nature, and existing selection pressures have filtered out the most successful gene/bacteria combinations for the present-day bacterial population, including the pathogens that now exist. This is an on-going process and future events of hgt can shift the virulence of present-day pathogens. When such events happen in nature all the time, the question could be asked what the increased risk is of horizontal gene transfer that occurs in the laboratory. The differences between 'natural' and experimental hgt is the subject of the next section.

4.6. Experimentally induced horizontal gene transfer.

Molecular genetics has provided powerful tools to identify and test virulence genes, as illustrated in Chapter 1. One of the strongest tools we have is complementation. In complementation experiments a gene is added to a bacterium that lacks a certain phenotype and it is tested if the addition of that gene can complement the genetic make-up so that this phenotype is now expressed. The wide-spread application of this technique demonstrates how powerful horizontal gene transfer can be in producing new phenotypes, including new or increased virulence in otherwise avirulent or less-virulent species.
Laboratory techniques allow gene transfers that are not possible under natural conditions, because certain restrictions can specifically be overcome. The most important restrictions that can be overcome are:

- Combining DNA of different species that occupy different niches outside the laboratory, and whose DNA would not normally be part of the same gene pool
- Forcing DNA into bacteria that are not naturally able to take up DNA and for which DNA transfer vehicles (phages, plasmids etc) are not known

- Restriction/modification systems that are overcome by using specific mutants [264]
- Incompatible plasmid combinations that are combined within one cell by the introduction of specific mutations
- Overexpression of genes that use rare codons by the addition of extra tRNA copies on plasmids

Such experimental procedures help to cross natural boundaries of horizontal gene transfer and to increase the stability and expression of such unnaturally acquired genes. They have been a powerful tool in the study of bacterial pathogenicity, and illustrate how horizontal gene transfer can produce new phenotypes in bacteria in seemingly infinite variations. The modern concept of molecular pathogenicity requires the complementation of deletion mutants of pathogens to fulfill Koch's postulates, and for this reason shuttle vectors are created for every possible pathogen (for example [264b]). It goes without saying that these experiments must be carried out with microorganisms that have retained their colonizing capacity.

There are few cases described in which virulence genes were intentionally introduced in another pathogen to assess the effect on virulence. Cloning of *Listeria monocytogenes* hemolysin (the presumed toxin that helps this intracellular pathogen to escape the phagolysosome) into *Bacillus subtilis* rendered this organism the capacity to grow in the cytoplasm of macrophages where it had been passively internalized [274]. These results show that a single gene product is sufficient to convert a common soil bacterium into a parasite that can grow in the cytoplasm of a mammalian cell. It should be stated, however, that the life style of the transformant is still a soil bacterium: it would probably not survive the selection pressure of existing microflora in the gut like *L. monocytogenes* can do. Similar results were obtained with cloning perfringolysin (the hemolysin from *Clostridium perfringens*) into *Bacillus subtilis,* but the cloning of streptolysin had no effect [275]. Expression of filamentous hemagglutinin (FHA) of *Bordetella pertussis* in *E. coli* is possible after some engineering for optimal translation [276]. Over-expression of botulin toxin in *Clostridium botulinum* poses no experimental difficulties with the use of shuttle vectors [277]. The risk that such genetically modified pathogens could pose when accidentally released in the environment must not be overlooked.

One direction of research is frequently ignored as potentially at risk for producing virulent organisms: the cloning of genes that encode biologically active molecules derived from the host into bacteria. Examples are: expression of cytokines in bacteria, such as human interferon gamma [265] or tumor necrosis factor alpha in *E. coli* [266]; the cloning of biosynthetic genes, e.g. taxadiene synthetase, the enzyme that produces taxol (a cytotoxin used for chemotherapy) in *E. coli* [267], or the cloning of hormones [268]. Since the effect of such biological factors on the host during unintended colonization is not known, the cloning and expression of biologically active factors (structural genes or biosynthetic enzymes) in bacteria should be treated with caution.

Chapter 5. Discussion

The aim of this literature survey on pathogenicity and virulence of bacteria was to provide a state-of-the-art review for the Ministry of the Environment (VROM), and to serve as a reference source for the Advisory Committee on Genetic Modification (COGEM). This committee is planning to re-evaluate the safety regulations for working with genetically modified organisms. The collected data will therefore be discussed within the scope of experimental genetic modification. Safety considerations of genetically engineered microorganisms that are released in the environment for agricultural purposes are outside the scope of this discussion.

5.1. Working with genetically modified organisms: new insights in bacterial virulence

The main criterion of safety regulations for working with pathogenic bacteria at present is their degree of pathogenicity. In the past, there have been concerns that genetically modified pathogens could be potentially more virulent than existing pathogens. Although the safety strategies have been sufficient and effective for more than 20 years (there has never been an outbreak of a disease caused by a genetically modified organism to date to our current knowledge), the present-day practice can be improved. At present, safety regulations are based on the taxonomic grouping of bacteria, and on the observed degree of pathogenicity of any member of the species in question. In other words, a bacterium is classified according to it's taxonomic group, and evaluated according to the most severe pathogenic properties observed with members of that same group. Cloning of DNA derived from this organism into another species results in a genetically modified organism that has to be handled at a certain degree of containment because of the genetic modification, on top of the biohazard of the original pathogen. Experimental DNA transfer between species is always regarded as artificial genetic modification but cloning between members of the same species (self-cloning) is not interpreted as artificial genetic modification.

Table 5

Incomplete understanding or difficulties in defining bacterial properties relevant for regulations on genetic modification of microorganisms

Drawbacks of defining virulence	**examples**
Toxin-producing, non-colonizing bacteria are not included in the definition of virulence	The genes encoding for botulin toxin are not included in the term virulence gene, however they have the potential to become virulence genes when they would be transferred to colonizing bacteria.
Virulent properties are seen as determined by the bacteria	The influence of the host is incorrectly ignored. The effect of the host susceptibility is especially important with opportunistic pathogens, since host-derived conditions determine when commensal bacteria become pathogens.
Drawbacks of identification of virulence genes	**examples**
Identification by phenotypic evidence of knock-out mutants depends on available (animal) models, is imprecise, identifies too many genes, and misses other genes of importance	Knock-out mutants of virulence-regulatory and virulence-associated genes can result in attenuation just like inactivation of primary virulence genes. Knock-out mutants of primary virulence genes can be complemented by other genes without phenotypic effects. Inactivation of silencer genes can result in increased virulence.
Identification by comparative genetics depends on functional extrapolation which may be misleading	Sequence similarity is interpreted as identity in function, regardless of organism-specific adaptation of gene function. Unique virulence genes will not be identified in this way. Genes may have multiple functions in single organisms, or different functions in different organisms.
Drawbacks of evidence for horizontal gene transfer	**examples**
Phylogenetic evidence is often unreliable and data are often incomplete	The source of the transferred gene cannot be determined by phylogeny since the outcome of the analysis depends on the set of genes included in the analysis. Transfer of gene fragments (domains) is likely to be missed though they can have major effects on protein function.
Incorrectly, only horizontal gene transfer of virulence genes is regarded relevant for virulence	The transfer of other genes may affect virulence, especially genes involved in expression and processing of virulence-associated genes, or genes encoding antigenic factors

There are a number of reasons why these regulations are in need of improvement. First, it has been suggested that the introduction of engineered bacteria to the environment is being over-regulated and that "advances [are] hampered by clumsy regulations based on unsubstantiated guesses" [269]. Second, it is questionable whether taxonomic classification is the most reliable and optimal framework for the safety classification of this kind of experiments. Third, since we have seen that bacteria exchange genes in nature, the risk of experimental genetic modification should be evaluated against the backdrop of events that happen in nature, resulting in similar exchange. Fourth, the methodological revolution in molecular biology has increased and shifted the potential of experimental possibilities since the time that genetic modification came into practice. Fifth, knowing that DNA can be integrated by microorganisms spontaneously, the DNA of genetically modified organisms can pose risks even after the microorganisms are killed. For these reasons it is valid to reconsider the risk evaluation that is the basis for current legislation. The data collected from the literature presented in the previous chapters are all relevant to this discussion, but the complexity of the information is overwhelming. For clarity, a number of observations that conflict with current legislation are extracted and presented in Table 5. These conflicts are either due to incomplete current knowledge, new insights, and/or narrow-minded interpretation of the data. In addition to the drawbacks mentioned in Table 5, the following observations need to be considered:

- Virulence and pathogenicity are often addressed in an anthropomorphic manner. This leads to the incorrect concept that pathogenic bacteria exist to cause disease in their host. Like every organism, pathogens have adapted to occupy their ecological niche. Their close association with a host causes damage to their host. Often this damage is 'coincidental', but it may even be beneficial to the survival or spreading of the pathogen (for example liberation of nutrients by cell damage, or enabling contagion of the next host by inducing coughing or diarrhea). The degree of damage is dependent on the equilibrium that results from the interplay of pathogen and host. The conditions that result in disease can vary between individuals, and from host species to host species. For these reasons it is not always possible to identify the bacterial genes that are directly responsible for the disease.

- Pathogens are constantly evolving, because the bacterial and the host population, as well as the ecological conditions that provide the interplay of both, undergo constant changes. Pathogens emerge and loose significance over time. The pathogen *Legionella pneumoniae* became a problem only after the wide-spread use of hot water systems (e.g. showers) and air-conditioning. The use of invading medicine enabled *Staphylococcus epidermis* to become pathogenic. Rodent control decreased the spread of *Yersinia pestis.* These examples illustrate that pathogenicity is the outcome of an intricate process with many variables. It is an oversimplification to blame bacterial virulence genes exclusively for pathogenicity.
- Bacterial taxonomy describes the genetic and biochemical similarities and differences of bacterial species. The borders that define a bacterial species are based on similarity in morphology and biochemical and physiological characteristics, homology between conserved DNA sequences, like ribosomal RNA genes, and on over-all DNA-DNA hybridization kinetics, as a measure for general genetic similarity. This definition is fundamentally different from the definition of a species that is used for higher organisms that is based on sexual reproduction and fertile offspring. Exchange of genetic information between different bacterial species is the rule rather than the exception. The taxonomic classification of bacteria is a rapidly developing field, where species are frequently reclassified, and genera redefined, created, or combined. Despite the use of standardized criteria, some bacterial taxa still reflect historical choices. For instance, the genus *Escherichia,* with it's most important member *E. coli*, should by modern definition include *Shigella* in it's genus, whereas the genetic difference between different *E. coli* strains would suffice the creation of a new species. In addition to the frequent updating of taxonomic classification and the existing inconsistencies, it should also be noted that bacterial isolates within a given species can differ considerably in degree of pathogenicity. Again, *E. coli* is exemplary because pathogenic *E. coli* strains belong to the same species as commensal *E. coli.* Another example is *Salmonella enterica* where many serovars represent different pathotypes. For these reasons taxonomic classification alone is not a proper tool for defining or predicting the pathogenic potential of an organism. It is equally important to evaluate the virulence potential of the strain, clone, variant, isolate, etc. in question.

- Bacteria can share genes in nature, but this is not a random process. Gene sharing requires transfer and subsequent fixation of DNA. Sharing of certain genes can be observed more frequently in certain species, either because their transfers are more frequent, or because the required fixation and expression is more successful. Frequent genetic exchange among certain species results in a number of genes that are shared by these species, comprising the gene pool of that group of organisms. Horizontal spreading of genes is further limited by natural barriers, that prevent complete genetic homogenization. Such barriers can be physical, for instance differences in ecological niches that do not allow bacteria to meet and mate, or biochemical, for instance restriction/modification barriers. Even when transfers occur, the transferred genes may not be fixed in the population because they do not provide an advantage for the accepting organism. Genes that provide high selective advantage will spread most efficiently, overcoming even high natural barriers.
- Under laboratory conditions, genetic transfer between species that would not exchange DNA under natural conditions is feasible. The naturally occurring limitations that prevent or restrict DNA transfer in nature can be overcome, and organisms that do not take up DNA naturally can be made to do so under laboratory conditions. Even when transfer of DNA between given species does occur in nature, the frequency of transfer can be increased by several orders of magnitude by experimental procedures. Barriers that prevent expression of genes in certain organisms can be overcome by artificially overruling regulation of transcription, translation, and expression. For these reasons, the soothing argument of 'self-cloning' ("DNA transfers that can take place under natural conditions do not pose an increased risk when applied in the laboratory") can be misleading.
- Genes and gene fragments can be amplified independent of the organisms they were created in, provided they are taken up by other organisms, in the laboratory or in the environment. Thus, the risk of genetically modified DNA should not only be assessed in the context of the organism it will be part of, but also in an unpredictable new genetic background. The chance of DNA fragments released in the environment to be taken up and integrated in new hosts is unknown. The observation that virulence genes are often beneficial to the growth rate of a pathogen, asks for serious measures:

virulence genes do not only cause disease, they may also give their hosting organism the best chance of survival [270].

In conclusion, the concept of virulence as mainly dictated by specific properties of a pathogen, that can be predicted by taxonomic relationship, that is shared between members of the same species, and that can be transferred to another bacterium to produce a new pathogen, should be revised. An alternative approach to estimate the risk of genetic modification of (pathogenic) bacteria is to incorporate the concepts of bacterial life-style and gene pools in the risk assessment. Such an approach would evaluate the ecological niche of the organism, identifying organisms that live in close association with a host as potentially at risk to gain virulence. The risk of producing potentially virulent bacteria is not predominantly determined by the pathogenic properties of the donor strain, but rather depends on the type of life-style of both organisms, with emphasis on the recipient of transferred DNA. If that acceptor strain is not fit to colonize or survive in a host, the risk of creating virulence is neglectable. As will be discussed in this chapter, the risk of exchange of DNA does not depend on 'natural vs. non-natural' transfers, nor on the distinction between 'self-cloning' and 'artificial' DNA introductions. The crucial determinants for risk assessment are bacterial life-style and biohazard. The risk of crossing gene pools that are available to a bacterial species is discussed in section 5.4 and opposing interpretations are presented in section 5.5 with illustrative test cases. First, an interpretation of the risks involved in genetic modification of organisms with an apathogenic life-style (5.2) and organisms with the ability to live in close association with a host (5.3) is given. The safety evaluation based on life-style, combined with the biohazard of the organisms involved, is presented in section 5.4.

5.2. Interpretation of risks of genetic modification of bacteria with an apathogenic life-style

The risk of producing virulent bacteria by genetic modification is reduced to acceptable levels when the acceptor strain does not have the ability to maintain itself in close association with a host. This is the case with bacteria that fail to survive and compete in

the ecological niches that mammalian surfaces provide. The observation that such bacteria could cause disease when they are artificially introduced into a body (for instance by contamination of incisive medical instruments) should not be taken into account. Their life style makes them unfit to independently compete with natural microflora in mammalian niches, to colonize, and thus to cause disease. An attenuated and laboratory-adapted strain derived from a pathogen that has such auxotrophic growth requirements that it has no survival chances outside the laboratory also fall in this category. These bacteria collectively have an 'apathogenic life-style'.

Bacteria with an apathogenic life-style can not colonize persons who get infected by accident. The risk of their spread in the host or in the environment is low [271], though survival in the environment may not be impossible [272]. However their genes can survive, because they can be passed on to other organisms even after death. The risk of spreading cloned DNA by attenuated host bacteria that function as a portal should not be ignored: sufficient decontamination measures should be taken to destroy both microorganism and DNA before release or disposal.

When DNA, derived from pathogens, is cloned into apathogenic life-style bacteria, it is not expected that this will result in pathogenicity, as the addition of (a) primary virulence gene(s) is not sufficient to change the life-style from apathogenic to (potentially) pathogenic. In order to become pathogenic, those bacteria also need to gain all properties required to colonize the host, compete with existing microflora, and survive non-specific host defenses. Even when large DNA fragments are obtained from pathogens, e.g. when cosmids are cloned, the risk of increased virulence of such an acceptor strain is neglectable. For this reason, experiments in which the acceptor strain has an 'apathogenic life-style' are regarded as low-risk. However, when these bacteria pass on their cloned DNA to others, the requirements for virulence may be met. For this reason, containment measures should be sufficient to prevent release of living organisms in the environment. If possible, mutations in the host strain, for instance mutation of conjugation potential, can diminish the risk that the newly-acquired genes could be passed on to other bacteria. Even so, unwanted horizontal gene transfer can not be ruled out completely. Therefore, decontamination methods that destroy DNA are to be preferred over those that only kill the bacteria.

The new insights in regulation and expression of bacterial virulence do not prompt an enforcement of the safety regulations for working with non-colonizing bacteria or laboratory-attenuated strains that have lost their ability to colonize, in which DNA is cloned that is derived from apathogenic or pathogenic organisms. On the contrary, our current knowledge of the intricate regulation and expression systems involved in bacterial virulence would allow relaxation of safety measures for such experiments, but the decontamination methods need to be reconsidered. The life-style of the recipient strain should be the first determinant for safety measures.

5.3. Interpretation of risks of genetic modification of bacteria with a pathogenic life-style

All bacteria that have the ability to live in close association with warm-blooded animals have the potential to develop into a pathogen. Zoonotic pathogens are exemplary of bacteria that can live in commensialism in certain animals, and cause disease in humans or other animals. Bacteria with a life-style that allows survival on mucoid surfaces have the potential to become pathogenic even if they are not pathogens in the classical meaning (commensals), ore if they are made apathogenic (attenuated mutants), by accumulation of the proper virulence factors. These bacteria are collectively described here as having a 'pathogenic life-style', regardless of their pathogenic properties at present: they have the potential to become pathogenic. Below are listed the most common experiments involving genetic modification of bacteria belonging to this group.

Producing and testing knock-out mutants of pathogens

Modern research to elucidate pathogenic mechanisms often requires in vivo studies, in which pathogenic organisms are genetically modified and their virulence potential is tested in an animal model. Obviously, such experiments can only be carried out with organisms that are able to colonize, and, in most instances, have the potential to cause disease. Frequently, research is aimed at identifying virulence factors, and as discussed in section 1.4, this is often done by inactivation of potential or putative virulence genes. Such 'knock-out' mutants are often less virulent than their parental wild type, or at most

equally virulent, but do not, in general, have increased virulence potential. For such studies the safety measures need not exceed those applied to the pathogenic wildtype bacteria (as dictated by their biohazard). When the bacterial strain used for knock-out experiments is known to be decreased in virulence compared to fresh clinical isolates (for instance due to artificially introduced mutations) prior to the knock-out procedure, it could be argued that a relax of safety regulations, in respect to the biohazard, can be considered. The putative risk of spreading antibiotic resistance genes, which are often introduced in the genome during the knock-out procedure, is not discussed here.

Cloning of virulence genes between colonizers and/or pathogens

The mode of action of a virulence gene can best be studied in a disease model in which the interplay between host and bacteria is mimicked. In order to study specific virulence strategies, a complete set of all required virulence genes from a given pathogen can be cloned into a bacterial acceptor that does not normally has those virulent properties (virulence complementation). This can only work when the acceptor strain a priori is able to colonize the preferred site of the host. In other words, the donor DNA is derived from a pathogen, and is to be cloned into another species with a 'pathogenic life-style'. Here it could be argued that 'new' strategies or combinations of virulence could potentially originate from the DNA manipulation. The same is true in those instances when virulence genes from one pathogen are cloned into another pathogen. It should be mentioned that such experiments are not common, but that they are not experimentally impossible. These cases are the most questionable in terms of a possible risk of 'producing' virulence. Not all experiments that follow this general principle pose an equal risk: this clearly depends on the biohazard of the organisms involved.

5.4. Layers for determining risks of genetic modification of microorganisms

Genetic modification of microorganisms that possess virulence potential, or cloning of DNA derived from such organisms, is considered potentially at risk to enhance or produce virulence. Ideally, classification of the safety of such experiments should be based on the following criteria: virulence potential of the acceptor strain and of the donor

strain; the life-style of the acceptor strain; the role of identified virulence factors in the lifestyle of the donor strain, and the chance of artificial exchange of genes within or between gene pools. This would lead to the unworkable situation where experiments can only be assessed case by case. Obviously some degree of generalization is required, based on assumptions. The decision on the safety level of a proposed experiment could be enlightened by a strategy of stacked choices, in which three characteristics are discussed here: the life-style of the organisms involved (layer 1); the biohazard of those organisms (layer 2); and the gene pools to which the organisms belong (layer 3). Arguments will be presented below that the third layer, the gene pools, are irrelevant in terms of virulence risk-assessment.

For genetic modification experiments in which DNA is transferred from one organism to another, the pathogenic life-style of both donor and acceptor need to be considered, though not necessarily to the same degree: if the recipient strain has an 'apathogenic life-style', the pathogenicity of the donor is not relevant. If the recipient strain has a 'pathogenic life-style', then the pathogenicity of the donor strain is of importance. The virulence genes of this donor should not be treated different from its other genes. Suppose a PCR fragment, obtained from chromosomal DNA, is cloned directly into another organism. It can never be excluded that unknown contaminants are present that were obtained from elsewhere on the genome. Moreover, virulence genes are not always (correctly) identified, and the experimentally produced combination of genes can have unpredictable effects on virulence. Therefore, the safest approach is to consider the biosafety of the donor strain as a whole, and not to restrict the risk assessment to the gene(s) that are meant to be transferred, unless the exact nature of the fragment is determined by DNA sequencing. Once an insert has been sufficiently characterized, recloning of this into a new genetic background may be reclassified at a lower scale when appropriate. In conclusion, it would be wise to consider the complete potential of the donor strain and not just the potential of the gene(s) that are meant to be transferred.

Layer 1: the life-style of the genetically modified organisms.

Is it safe to neglect pathogenic properties of a donor strain when transferring its DNA into a recipient with an 'apathogenic life-style'? To evaluate the risk of creating virulent

properties in such a scenario, the multi-step process should be considered that is required to produce a pathogen from an apathogenic species. This involves the adaptation to living in close association with the host, competing with existing micro-flora, escaping the defense strategies of the host, and causing damage to the host. Bacteria that do not live in or on a host do not have these properties, and will not be fit for a life in a host even if it would acquire multiple genes from a pathogen. Commensal bacteria (which are colonizing but not pathogenic) have already adapted to this life style, and for this reason genetic manipulation of such bacteria is more at risk to create an unwanted virulent phenotype than bacteria that do not live in close association with a host. Thus, the target organism (the acceptor of DNA) should be classified according to its life-style with relation to the ecological niche it occupies. The first step in the level of safety-assessment based on life-style is therefore the life-style of the recipient organism, and the consequences of this approach are summarized in Table 6. The suggested classes are based on the probability that an organism with a given life-style can gain sufficient potential to become a pathogen to warm-blooded hosts. The second step in safety assessment at this level, and only applicable when the recipient strain has a pathogenic life-style, is the life-style of the donor organism (see Table 6). An additional group of donor organisms (including eukaryotes) is specified that produce biologically active molecules, such as toxins, hormones, or cytokines, either by nature or by artificially introduced genes. Cloning of biologically active molecules derived from eukaryotes (via cDNA) is explicitly included here. In experiments where the DNA of the original microorganism is modified without the addition of foreign DNA the proposed guidelines for 'recipient' should be applied. The major differences of this proposal with current regulations are: (1) genetic modification of bacteria with an apathogenic life-style does not require additional safety measures; (2) all experiments involving genetic modification require destruction of microorganisms and DNA before disposal/release; (3) genetic modification of bacteria with a pathogenic life-style need to be assessed by level 2, even when cloning is performed between bacteria belonging to the same taxonomic class.

Table 6. Proposed classification of organisms based on level 1: bacterial life-style

Life-style of the acceptor strain	**Description**	**Possible refinements**	**Consequences**
Apathogenic life-style: extremophiles e.g. cryophile, thermophile, halophile, etc.	Grow only at conditions excluding conditions encountered in warm-blooded hosts		Genetic modification of these bacteria does not require extra safety measures, regardless of the incoming DNA, other than destruction of microorganisms and DNA before disposal/release.
Apathogenic life-style: non-colonizing bacteria e.g. soil bacteria	Grow in environment without association to a host, no pathogenicity documented		
Pathogenic life-style: commensal colonizers	Colonize a host as a commensal	Since conditions determine if a commensal becomes pathogenic these classes can be combined further classification according to biohazard	Genetic modification of these bacteria requires safety measures depending on the biohazard of the acceptor strain and the donor strain (level 2).
Pathogenic life-style: opportunistic pathogens	Colonize and cause disease under certain circumstances		
Pathogenic life-style: pathogens	Causes disease in every susceptible host		
Life-style of the donor strain	**Description**	**Possible refinements**	**Consequences**
Apathogenic life-style bacteria (see above)	Is never found in association to a host		Virulence can not be transferred. Extra safety measures are not required.
Pathogenic life-stylebacteria	Colonizes a host and may or may not cause disease	Colonizers, opportunistic pathogens, and pathogens can be separated; biohazard of the latter should be included	Transfer of DNA to apathogenic life-style bacteria does not require extra safety measures Transfer to other pathogenic life-style bacteria poses a risk of increased virulence. Safety evaluation depends on level 2.
producers of biologically active factors	no colonization potential but producing factors with a potentially damaging effect to mammals		Transfer of DNA into pathogenic life-style bacteria requires extra safety measures.

Layer 2. The biohazard class of pathogenic bacteria.

It is obvious that not every pathogen deserves the same caution for experimental use. The biohazard of pathogens depends on their pathogenic potential, their morbidity and mortality, and risk and mode of spread. Currently used biohazard classifications [273] can define the criteria for this layer of risk assessment. Both the biohazard of the acceptor and the donor strain are of importance, provided the acceptor strain has a pathogenic life-style. For reasons discussed above, it is advised to consider the biohazard of the organism, and not of the genes that are aimed for the transfer. The biosafety level should then be dictated by the organism with the highest biohazard, regardless whether this is the acceptor or the donor, until the genes that were transferred are characterized sufficiently to allow a relax of safety measures.

When the donor DNA is encoding biologically active factors, the biosafety of the acceptor strain should be considered in combination with the predictable effects of these factors. When no DNA donor is involved (genetic modification without the introduction of foreign DNA) the biosafety level is determined by the biohazard of the original bacterium.

Layer 3. The gene pool.

How important is the distinction between DNA transfers that are in principle possible in nature (within one gene pool) and transfers that cross natural boundaries (between gene pools)? It is possible to identify groups of bacterial species that share genetic information under natural conditions, by phylogenetic analysis of their known genes. The growing number of complete microbial genome sequences, and of individual gene entries in public databases enables the identification of such gene pools. However, sequencing data of microbes are still highly incomplete, and though databases are growing rapidly, completeness will never be achieved.

A crucial observation is, that in nature horizontal gene transfers take place that result in increased virulence potential. It can therefore not be assumed that gene transfer within a gene pool is without risk of increasing virulence potential. It should also be noted that experimental gene transfer can overrule natural barriers of transformation, gene regulation, and expression, with unpredictable outcome, even when such experimental

gene transfers involve members of the same gene pool. In other words, experimental DNA transfer within a gene pool can have stronger effects than natural transfers.
When it was recognized that certain bacterial species can share their DNA under natural conditions the term 'self-cloning' was introduced. The argument was used that processes that take place in nature are not in need of extra containment when employed in the laboratory. Meanwhile, this view is in need of re-interpretation. The efficiency of experimental transfers exceeds that of natural transfer significantly, and from the study of the evolution of pathogens the important role of natural DNA transfers in producing virulence has become evident. One could argue that artificial DNA transfers between members of one gene pool have a better chance of increasing virulence, since these members have already proven to be able to 'use' each others genes. Complications that limit expression of incoming genes, like insufficient gene regulatory mechanisms, improper codon usage, etc. are more likely to be absent. Therefore, gene transfers between members of one gene pool are at risk of producing or increasing virulence.
On the other hand, artificial gene transfer between members of different gene pools allows genes crossing borders that would not naturally be crossed. In that case the outcome is hard to predict, but an increase in virulence can not be excluded when genes are involved derived from bacteria with a pathogenic life-style.
The conclusion must be that gene transfer between and within gene pools both have the potential risk of increasing virulence, provided the pathogenic life-style of both species. The consequence is, that self-cloning should not be treated differently from DNA transfers that are only possible under experimental conditions. It is irrelevant whether experimental DNA transfers take place between or within gene pools, or between species or within strains of the same species. Relevant is the life-style of the organism in combination with the biohazard.
The validity of the argumentation discussed above is a matter of interpretation: how should the risk be assessed of an experiment in which DNA is transferred within or between gene pools? The different interpretations of this risk can best be illustrated with hypothetical experiments as outlined below: listed in Section 5.5 are conflicting interpretations of hypothetical cases with their arguments in favor, and the disadvantages or unwanted consequences against each of them.

5.5. Interpretations of the risk of gene transfers within and between gene pools

Case A: A pathogen contains one or more genes of which it has been proven that these were obtained by naturally occurring processes. A second pathogen that belongs to the same gene pool is planned to be used as donor for DNA that is to be cloned in the first.

Interpretation (1) of case A: This experiment DOES NOT require additional safety regulations (see footnote[1])

- Arguments in favor of interpretation (1):
  - The accepting bacterium has the natural ability to accept DNA in nature, and this is exploited under experimental conditions. The modification applied in the laboratory is not essentially different from the processes that take place, or have taken place, in nature.
  - Under the circumstances mentioned, regulations and restrictions are useless because they can not be checked. Even when the result is an increase of virulence, retrospectively it can be difficult to proof if virulence genes have been acquired by experimental modification or by natural processes.
- Consequences and disadvantages of interpretation (1)
  - DNA transfers under laboratory conditions occur at much higher frequency than in nature and can be forced into a certain direction: they are not the result of pure chance. For bacteria of which the complete genomic DNA sequence is not available it is not known whether they contain exogenous DNA. The criteria to identify genes obtained by gene transfer are not exactly defined.

Interpretation (2) of case A: This experiment DOES NEED additional safety regulations

- Arguments in favor of interpretation (2):
  - When pathogens have already proven to be able to take up and exploit exogenous DNA, it is not unlikely that such a process leads to an increase in virulence at some stage. The consequences of such an event cannot be predicted.

[1] Note: with additional safety regulations, those regulations are meant that surpass the regulations for working with this pathogen without genetic modification.

    - Under laboratory conditions, natural barriers for horizontal gene transfer can be overcome, so that the efficiency of transfers is much higher than those in nature. Gene expression can be artificially increased.
- Consequences and disadvantages of interpretation (2)
    - It will be hard to accept for scientists that experimental actions that can take place in nature unlimited and uncontrolled, need to be carried out under extra safety regulations in the laboratory.

Interpretation (3) of case A: Our present-day knowledge about the genetic background of bacterial virulence, and about the role and importance of horizontal gene transfer for the evolution and spread of virulence genes, is not sufficient to justify a risk assessment of potential virulence increase after genetic modification of pathogens

- Arguments in favor of interpretation (3):
    - Our knowledge of virulence genes is fragmented and incomplete. Many (emerging) pathogens are not sufficiently characterized and our knowledge is biased and simplified by experimental restrictions. Variation in virulence and gene content existing between strains belonging to the same species is not sufficiently validated. For these reasons it is often impossible to identify those genes that contribute most to the virulence potential of a given pathogen.
    - Pathogenicity is an interplay between bacterium and host. The latter has such an impact on the resulting disease, that it is incorrect to completely blame bacterial genes for this. Moreover, in the gray zone of commensals and opportunists, a gene without obvious virulence potential can cause a shift towards pathogenicity in a susceptible host.
- Consequences and disadvantages of interpretation (3)
    - By lack of alternatives the legislation of working with genetically modified microorganisms will remain unchanged, despite recognized flaws.

The last interpretation does not help in developing alternatives to current regulations. When the choice is between the first interpretation and the second, the latter would be my favorite. The argument in favor of interpretation (1) is weak: although experimental DNA transfer is not essentially different from natural transfer, the resulting frequency is. The argument against (2), that scientists would not accept regulations of processes that occur

in nature is not convincing. Taking the second as the best, this would mean that genetic modification of pathogenic organisms in which 'self-cloning' is employed, or, more precisely, in which DNA is exchanged between organisms belonging to the same gene pool, is regarded as potentially at risk to produce mutants of enhanced virulence. It should be stressed that in this case the organisms involved are pathogens or have a pathogenic life-style. The risk of producing virulence by genetic modification of apathogenic life-style bacteria is neglectable, as discussed in section 5.2).

Case B. A pathogen contains one or more genes of which it has been proven that these were obtained by naturally occurring processes. In contrast to the case described above, the DNA cloned into this organism is derived from a second pathogen that belongs to a different gene pool.

Interpretation (1) of case B: This experiment DOES NOT require additional safety regulations as compared to case A.

- Arguments in favor of interpretation (1):
    - The fact that both bacteria belong to different gene pools means that they probably cannot incorporate each others gene products in their existing physiology. The chance that an artificial DNA exchange of this kind will result in increased virulence is expected less likely than in case A.
- Consequences and disadvantages of interpretation (1)
    - This interpretation is a paradox: the longer the genetic distance between two bacteria, the less likely would these share their genes by natural processes. But this does not exclude the possibility that the incoming DNA will be functional. When DNA exchange is achieved artificially between such organisms there is no reason to interpret this as 'less risky' than transfers that could occur in nature.

Interpretation (2) of case B: This experiment requires ADDITIONAL safety regulations as compared to case A.

- Arguments in favor of interpretation (2):

- This is a completely artificial and unnatural genetic transfer with unpredictable outcome. The risk of such transfers for creating or increasing virulence can never be assessed. Therefore, extra safety containment is required.

- Consequences and disadvantages of interpretation (2)
  - Evolution proceeds with small steps; bacterial virulence evolves with many minor achievements. It is hard to believe that a 'superbug' would be formed when the DNA of two completely non-related organisms was combined.

It is hard to decide which interpretation is best, since experimental data in favor of the one or the other is lacking. My choice would be that in case B extra safety regulations are required because unwanted effects on virulence can not be excluded. The same is true in the following case:

Case C. A pathogen contains one or more genes of which it has been proven that these were obtained by naturally occurring processes. It is planned to clone DNA into this organism that codes for a factor with known biological function.

In this case the gene that will be introduced could encode a toxin, a hormone, or a cytokine. This may or may not be an 'unnatural' transfer (exceeding gene pool boundaries), but my choice would be:

Interpretation (1) of case C: This experiment requires ADDITIONAL safety regulations.

- Arguments in favor of interpretation (1):
  - This is a completely artificial and unnatural genetic transfer with unpredictable outcome. The risk of creating an organism with unknown biological effects cannot be ignored.

It is outside the scope of this project to make a decision in which direction future legislation should go. The choices presented here are the personal opinion of the author. Whichever interpretation is followed, observations described in the literature of experiments conducted in the past could be used as a tool for retrospective risk assessment, before new rules were to be put to practice. The examples of natural exchange of virulence genes collected in this study will provide a basis for such exercise.

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