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JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GUIDANCE DOCUMENT ON THE USE OF INFORMATION ON PATHOGENICITY FACTORS IN ASSESSING THE POTENTIAL ADVERSE HEALTH EFFECTS OF MICRO-ORGANISMS: BACTERIA

Series on Harmonisation of Regulatory Oversight in Biotechnology No. 52

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No. 52

Guidance Document on the Use of Information on Pathogenicity Factors in Assessing the Protential Adverse Health Effects of Micro-Organisms: Bacteria

Environment Directorate

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FOREWORD

The OECD's Working Group on the Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of *consensus documents* which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product.

On reviewing a published consensus document and drafting other consensus documents on micro-organisms, the Working Group felt that these documents did not focus in a straightforward way on questions that are relevant to risk/safety assessment issues.

Responding to the concern, the Working Group decided to take an alternative approach, namely the development of *guidance documents* in the micro-organisms area which are intended to provide guidance on specific topics and issues that are relevant to risk/safety assessment in biotechnology. The Working Group also established a sub-working group that consists of experts on micro-organisms and focuses on projects related to the safety of environmental applications involving micro-organisms, especially bacteria. Subsequently, the sub-working group has developed several useful documents for environmental risk/safety assessment of micro-organisms within the Working Group.

This guidance document addresses the concept of bacterial pathogenicity in the context of risk/safety assessment of deliberate release of 'genetically engineered', or 'genetically modified' micro-organisms intended for commercial environmental applications. It explores the factors that are important in bacteria for causing adverse human health effects and assesses how this knowledge can be used in risk/safety assessment of environmental applications of bacteria. It is primarily intended for use by risk assessors, but may also be useful for applicants and other stakeholders in the regulatory process.

The Netherlands and Canada served as lead countries in the preparation of this document, cooperating with the sub-working group. It has been revised on a number of occasions based on the input from other member countries and observers.

This document was endorsed by the Working Group on the Harmonisation of Regulatory Oversight in Biotechnology at its 25th meeting held of the OECD Headquarters on 9-10 May 2011. It is declassified and published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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SECTION I - GENERAL CONSIDERATIONS FOR BACTERIAL PATHOGENICITY

1.   This document provides guidance on the concept of bacterial pathogenicity in the context of risk/safety assessment of deliberate release of 'genetically engineered', or 'genetically modified'[1], micro-organisms intended for commercial environmental applications (*e.g.* bioremediation, biosensors, biofertilisers, biopesticides, biomining, biomass conversion, or oil recovery). It is limited in scope to bacteria that may exhibit properties pathogenic to human beings. Not included in the scope are environmental releases of known (potential) pathogens, *e.g.* vaccine strains. The document explores the factors that are important in bacteria for causing adverse human health effects and assesses how this knowledge can be used in risk/safety assessment of environmental applications of bacteria. Where appropriate, the document also refers to certain aspects of mamalian bacterial pathogens. For specific aspects of plant and/or other animal (*e.g.* fish, insects, and other invertebrates) pathogens, separate documents on these issues would be needed.

2.   Genetically engineered bacteria applied for environmental purposes, including field trials, should be evaluated to determine whether they may pose hazards to human health, which this document addresses. The analysis from the OECD 'Blue Book' on recombinant DNA safety (OECD, 1986) appears to be still valid: Agricultural applications may result in release of large quantities of modified [micro]-organisms into terrestrial or aquatic ecosystems. Recombinant DNA-derived vaccines for animals and humans, as well as certain plant-associated micro-organisms, may in some cases have a limited pattern of environmental exposure because of biological specificity to the host, but incidental release to the environment certainly occurs in sewage and feed-lot or run-off waters, and may be significant. Environmental applications (*e.g.* metal extraction, pollutant and toxic waste degradation) may be confined initially to a specific location or may result in broad ecosystem exposure. The scientific considerations for assessing risk/safety will vary with each particular environmental application, depending on the organism, the physical and biological proximity to man and/or other significant biota. Local quarantine regulations, confinement measures, and monitoring methodologies utilised during research and development will also be relevant.

3.   In general, prior to their release bacterial strains should be submitted to an assessment of their potential health effects, including their pathogenicity. As 'virulence' is the quantitative measure of the pathogenicity of a micro-organism, the virulence factors of a bacterial strain are its traits that will be taken into account in the risk/safety assessment. For the special case of genetically engineered micro-organisms the risk/safety assessment should take into account any characteristics of the engineered micro-organism related to pathogenicity, and whether any introduced traits are associated with pathogenicity.

4.   When performing a regulatory review of the role of a donor gene as a virulence factor in the recipient micro-organism, regulators need a good understanding of the significance of a given virulence gene in the physiological background of the donor organism, as well as of the constitutionof the recipient micro-organism. A large number of interacting factors affect the ability of a micro-organism to become pathogenic, and acquisition of a single gene in the absence of other genes necessary for pathogenicity will

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[1] The terms 'genetically engineered micro-organism' and 'genetically modified micro-organism' are used in different legislative systems for micro-organisms in which genetic information has been added or removed by techniques of modern biotechnology.

not likely convert a non-pathogen to a pathogen. Only if the newly acquired gene can have a role in the pathogenicity of the recipient micro-organism can an interaction be expected between the newly acquired gene and the resident genes contributing to a pathogenic lifestyle.

5.   Pathogenicity is a multifactorial process which depends on the immune status of the host, the nature of the bacterial species or strain, and the number of organisms in the exposure. Therefore, the risk/safety assessment for human health can only be done on a case-by-case basis, taking into account the activity(s) of the introduced gene(s), the (potential) health hazards of the bacterial strain depending on the route of exposure (*e.g.* ingestion, inhalation, dermal contact), and the actual way that exposure to the strain is expected to occur under the conditions of the release. Exposure can depend on a number of factors, including the pattern of release (*e.g.* aerial spray, ground application, deep well injection, application into water bodies or effluent streams, shedding from inoculated humans or animals) and the scale of use (*e.g.* pilot, field trial, commercial use)

6.   Because this document is intended as an aid to general risk/safety assessment tool, its nature is generic, *i.e.* not organism specific, and refers to specific bacteria and characteristics only to illustrate specific concepts. In addition to describing potential adverse health effects, and the bacterial factors that can contribute to these effects, the document describes general considerations in assessing the potential hazard of un-modified bacteria, *e.g.* a description of some tools available for predicting pathogenicity. Lastly, the document addresses considerations for the potential to introduce or alter pathogenicity as a result of genetic modifications to the micro-organism.

1.1   General Considerations in Assessing the Hazardous Potential of Bacteria: The Concept of Bacterial Pathogenicity

7.   This section 1.1 and the next sections, 1.2 and 1.3, deal with the concept of bacterial pathogenicity in general, as it is discussed for unmodified bacteria; the concept also applies to genetically modified bacteria. Pathogenic bacteria have the ability to invade their hosts and produce disease. In this document, 'pathogenicity' is referred to as the property of a micro-organism to cause disease. The great majority of bacteria that are encountered in the environment usually do not present problems to human health, in the sense that no record exists of them behaving as pathogens. Many bacteria are even beneficial, *e.g.* because of their role in essential processes in the environment such as mineralization, or their function as human symbionts. There are many bacteria that may act as opportunistic pathogens, *i.e.* organisms that are normally present in the environment or as part of the commensal bacterial population of a host, but that may cause disease when defense systems of the host become debilitated, or when the equilibrium within the existing bacterial population is disrupted. In general, given the interplay between members of microbial communities and the interplay between microorganisms and potential hosts, it is unrealistic to say that a bacterium can never be a pathogen,, and probably 'non-pathogenic' bacteria can best be seen as bacteria that have not yet proven to have pathogenic potential.

8.   Although 'pathogenicity' can be defined in terms of properties of a micro-organism, it is important to keep in mind that the concept of pathogenicity is highly anthropomorphic, as it implies that a micro-organism would cause disease 'on purpose'. A more realistic view is that the body is a habitat for micro-organisms to adapt to and use as a favorable environment for survival and growth. Some bacteria have developed a 'lifestyle' that enables them to colonize this niche in symbiotic as well as in pathogenic ways (Wassenaar and Gaastra, 2001). Each body surface – skin, conjunctiva, mucous membranes of the upper and lower respiratory tract, intestinal tract, genital tract, and so forth – harbors a characteristic commensal bacterial population which differs qualitatively from the population of other areas of the body. Bacteria with pathogenic behavior may establish a foothold in this microbial ecosystem. Once established other pathogenic properties allow the pathogen to penetrate into deeper tissues, to avoid or counteract host defense mechanisms, and to multiply. As they pursue this strategy, pathogenic bacteria produce damage to

the host. Virulence-associated factors may be defined as all factors that are essential for expressing pathogenicity.

9.   Whether a host will develop disease is, however, not just determined by the pathogenic potential of the bacterium, but also by host factors. There are a formidable array of specific and non-specific host factors that affect the outcome of an encounter between a host and a pathogenic bacterium. For example, the normal commensal population plays an important role in protecting the host from invasion by pathogenic organisms. They do this by mechanisms such as (1) competition for the same nutrients; (2) competition for the same receptors on the host cells (tropism); (3) production of bacteriocins or other antimicrobial agents (interference); and (4) stimulation of cross-protective immune factors. The commensal population of the host may be affected by a number of activities (*e.g.* use of antibiotics). Additional host factors that can affect pathogenicity include the production of antimicrobial substances, (*e.g.* lysozyme in bronchial secretions, or the pancreatic enzymes, bile, or intestinal secretions, or secretion of acid (HCl) for low pH of the stomach). Also, humans have an innate immune system that protects against invasion. When this system breaks down, *e.g.* in advanced stages of acquired immunodeficiency syndrome (AIDS) (Gradon *et al.*, 1992), bacteria that are normally not able to cause disease in humans may become opportunistic pathogens that cause conditions that clinically mimic the more commonly encountered 'frank' pathogens. The potential of bacteria that normally occur in the environment to cause opportunistic infections in hosts with debilitated defense systems is recognized as an important human health hazard. The case of the *Burkholderia cepacia* complex (Bcc) is an example (Mahenthiralingham *et al.*, 2005). Bacteria of the Bcc are found throughout the environment, some as plant pathogens.

1.2   General Considerations in Assessing the Hazardous Potential of Bacteria: Classification of Risk Groups of Bacteria

10.   Pathogenic bacteria are commonly classified in risk groups, according to their pathogenic potential. The classification of the World Health Organization (WHO), as found in their Laboratory Biosafety manual (WHO, 2004), is generally accepted. It should be noted, though, that these risk groups are primarily concerned with laboratory applications, where exposure may be high. They are valid for persons that are not immunocompromised. According to this classification, risk group 1 ('no or low individual or community risk') comprises micro-organisms that are unlikely to cause human or animal disease. Risk group 2 ('moderate individual risk, low community risk') comprises pathogens that can cause human or animal disease but that are unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment; laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited. Risk group 3 ('high individual risk, low community risk') comprises pathogens that usually cause serious human or animal disease but do not ordinarily spread from one infected individual to another; effective treatment and preventive measures are available. Risk group 4 ('high individual and community risk') comprises pathogens that usually cause serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly; effective treatment and preventive measures are not usually available.

11.   For practical reasons, also in regulatory practice, a distinction is drawn between bacteria that are pathogenic to humans and bacteria that are pathogenic to other animals. Host specificity of bacteria is the result of differences between the environment that bacteria encounter in different hosts, *i.e.* in the human body and the bodies of other animals. If there are similarities between these environments it may be expected that pathogenic organisms frequently 'jump the species barrier'. Indeed, there are a number of bacteria that are primarily pathogenic to other vertebrates that are also pathogenic to humans, *e.g. Bacillus anthracis, Brucella abortus, Yersinia pestis, Leptospira* spp. and a number of *Salmonella* species. Human diseases caused by these bacteria are called zoonoses (see also Blancou *et al.*, 2005, for a review). In some cases insect vectors play a specific role in passing the pathogenic bacteria from

the animal to the human host. Zoonotic diseases are 'animal borne': animals, or animal products, act as a source of the disease. Consequently, exposure to the disease may change with changing social, behavioral and consumer practices. The risk class of a zoonotic bacterial species may differ depending on the host. For environmental risk/safety evaluations of activities with these bacterial species, the highest risk class has to be taken into consideration.

12.   As pointed out previously, it is difficult to definitively state that a bacterial strain is non-pathogenic. The evidence given for non-pathogenicity can only be tentative. The determination of whether a bacterial strain may be considered non-pathogenic is usually made in a stepwise fashion. The strain may be considered non-pathogenic if it belongs to a species or taxonomic group for which no pathogenic strains are known. If it has direct relatives that are pathogenic, or if it is derived as an attenuated pathogenic strain, it should be shown that the strain effectively lacks the virulence determinants of its pathogenic relatives. If this fails, evidence for non-pathogenicity can be obtained through appropriate animal testing. This requires however a validated animal model. If none of this evidence is available or can be obtained, the strain may be considered non-pathogenic because it has a long history of safe use under conditions where no specific physical containment, like a closed fermentor system, has been applied to reduce worker exposure.

13.   Although there is a clear value in using risk groups in practice,(*e.g.* refer to the WHO Laboratory Biosafety manual (WHO (2004), chapters 1 and 2), the concept of ' opportunistic pathogenicity' implies that there is a continuum from non-pathogens to full frank pathogens. Some bacteria complete their life cycle independent of a human or animal host. Others that lack the ability to cause disease may still be able to recognise, adhere to and multiply in or on the host, as commensals. Opportunistic pathogens have some limited ability to cause disease, but are normally kept under control by the host immune response and defense systems and the competitive, harmless micro-organisms with which they compete in the host's habitat. However, they may acquire a toehold, with adverse consequences for the host, generally under circumstances where the host defense mechanisms are compromised (*e.g.* weakening of the immune system through age or HIV infection) or destroyed (*e.g.* through skin lesions or burns). Some opportunistic pathogens are acquired from the environment while others may constitute part of the host normal bacterial population. Some bacterial species causing infections at hospitals are used in bioremediation and/or bioaugmentation processes that may involve inoculation of soil with large amounts of bacteria. For instance, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* are organisms used industrially that cause nosocomial infections in cystic fibrosis and burn patients. *Serratia marcescens*, a common soil bacterium, causes pneumonia, urinary tract infections, and bacteremia in compromised human hosts and is lethal to certain insect species with commercial use as a biopesiticde while commensal on the rhizoplane of many plant species. Other bacteria, such as *Lactobacillus acidophilus*, may be considered to be nonpathogens, because they rarely or never cause human disease. However, it should be noted that categorization as nonpathogens may change due to the inherent variability and adaptability of bacteria and the potential for detrimental effects on host defense systems caused for example by radiation therapy, chemotherapy, and immunotherapy, genetic defects (cystic fibrosis) or immunosuppressive infection (HIV).

1.3   General Considerations in Assessing the Hazardous Potential of Bacteria: Approaches to Bacterial Virulence

14.   In 1890 Robert Koch established his 'postulates', a standard for the evidence of causation in infectious disease. The evidence should show that (i) the micro-organism 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) after being isolated from the body and grown in pure culture, (iii) the micro-organism can be inoculated into a healthy host and induce the disease anew, and (iv) the micro-organism can be re-isolated after this experimental infection.

15.   Virulence factors can be defined in terms of Koch's postulates, as phenotypic properties of a micro-organism that are present in pathogenic strains that fulfill Koch's postulates, but that are not observed in related strains that are not pathogenic. Although the postulates have been generally accepted for over 100 years (Fredricks and Relman, 1996), Koch himself already recognized the limitations of these guidelines. For instance, the ability to cause disease as an invariant virulence trait has been challenged. In recent years in a more integrated view of microbial pathogenesis has been developed that recognizes the contributions of both the pathogen and its host are required. The lack of experimental models for human-specific pathogens limits testing of the third postulate, and consequently also the rigorous testing of the role of a human-specific virulence factor.

16.   Still, based on the notions of Koch's postulates a number of virulence factors have been identified because of their clear role in the pathogenesis or their clear-cut coincidence with pathogenic strains, (*e.g,* adhesins, invasins, haemolysins, or in general cytolysins). With the development of molecular biological techniques, it became possible to identify the genes encoding these known virulence factors and to identify genes of unknown function for which a possible role in virulence could be determined. This resulted in a new approach of research on bacterial pathogenicity, in which the role of specific genes in bacterial virulence was the key point.

17.   Virulence of a micro-organism is usually considered as the 'degree' of pathogenicity of the micro-organism in a susceptible host. Finlay and Falkow (1997) discussed the various definitions of microbial pathogenicity, and the idea that pathogens can be distinguished from their non-virulent counterparts by the presence of such virulence genes. A virulence factor is a phenotypic trait associated with the virulence level of a micro-organism. The term is also used for a gene product (or group of gene products) that is responsible for the phenotypic trait. Virulence factors add to the pathogenicity, by enhancing one or more of the processes involved in the stages of pathogenicity: (1) the ability of the bacterial pathogen to gain access to the individual by surviving on or penetrating skin and mucous membranes; (2) the *in vivo* multiplication of the pathogen; (3) the inhibition or avoidance of host protective mechanisms and (4) production of disease or damage to the host. In this document microbial toxins are regarded as virulence factors even though these toxins are defined as gene products[2] produced by a bacterium that can cause harmful effects in the absence of the active living bacterium because, in most cases the bacterium producing the toxin has to be established within the host in order to deliver the toxin most effectively. Therefore the phenotypic trait of toxin production may be seen as increasing the pathogenic potential of a bacterium, while full blown effects of a toxin may be dependent on other virulence factors of the producing micro-organism, (*e.g.* the ability to colonize the host). It should however be realised that some bacteria that are not regarded as pathogenic (*e.g.* neurotoxin producing cyanobacteria) may also produce toxins, and that some bacteria producing toxins that can act at a distance (*e.g. Clostridium botulinum* causing foodborne disease) are characterized as pathogens.

1.4   Outline of the Document

18.   This document provides an overview of the inherent bacterial components attributed to pathogenicity in section II, Bacterial Factors and Determinants for Pathogenicity. It deals in more detail with the genetics and molecular biology of bacterial pathogenicity in section III, Molecular Aspects of Bacterial Pathogenicity. Section IV discusses the problems of how to assess the potential pathogenicity of bacteria in an environmental risk/safety assessment of micro-organisms that are deliberately released, including genetically engineered bacteria.

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[2] Toxins may also be low molecular weight metabolites; this type of toxins is however not taken into consideration in this paper.

SECTION II - BACTERIAL FACTORS AND DETERMINANTS FOR PATHOGENICITY

19.   'Virulence' is a quantitative measure of the pathogenicity of a micro-organism that may be expressed by the ratio of the number of individuals developing clinical illness to the number of individuals exposed to the micro-organism, or in a comparative manner, by the number of individuals that develop clinical illness if the same dose of different micro-organisms is applied to each of them.

20.   Pathogenic bacteria have evolved a number of different mechanisms, which result in disease in the host. The virulence factors and determinants used by bacteria to interact with the host can be unique to specific pathogens or conserved across several different species or even genera. For instance common mechanisms for adherence, invasion, evasion of host defenses and damage to host cells are shared by profoundly different microbial pathogens. However, a virulence factor can only contribute to the pathogenic potential of a bacterium in and as far as the microorganism possesses the constellation of traits conducive to pathogenicity. This section examines bacterial factors/determinants that contribute to pathogenicity in bacteria. While these are the determinants that would generally be considered in a risk/safety assessment, it should be noted that the same factor/determinant will not necessarily have a similar effect on the virulence of two different bacteria and thus simple possession of a trait is not an indicator that the microorganism is pathogenic. The concept of the 'pathogenicity' of bacteria is further discussed in section III.

2.1   Host Recognition/Adherence

21.   Bacterial adherence to host surfaces is an essential first step in colonization, infection and disease production. Colonisation establishes the organism at the portal of entry. Whereas intact outer skin is generally impervious to invasion by organisms, surface penetration of the urogenital, digestive and respiratory tracts as well as the mucosal barrier is more easily accomplished. Much of the body that is usually regarded as internal is topologically connected to the exterior. For example, the surfaces of the intestinal lumen, the lung alveoli, the bile cannaliculi, and the kidney tubules are continuous with the outside skin. Organisms infecting these regions usually have elaborate adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses on the surface. Bacterial adherence to host cells is usually a prerequisite to invasion. Consequently, a great deal of research has focused on elucidating bacterial mechanisms of adherence to host cells (adhesin biosynthesis, regulation of adhesins, identification of host receptors).

22.   Adhesion can be defined as the coupling of a bacterium with a substratum. For molecules on the surface of the bacterium to interact with molecules on the surface of a host cell or the extracellular matrix (ECM), the two molecules must come into contact, an action that leads to the creation of intermolecular bonds requiring a certain amount of energy or effort to break. Bacterial adherence to a eukaryotic cell or tissue surface requires the participation of two factors: a receptor and an adhesin. The receptors so far defined are usually specific carbohydrate or peptide residues on the eukaryotic cell surface. Many bacterial adhesins are a macromolecular component of the bacterial cell surface which interacts with the host cell receptor. This interaction is usually complementary and specific, although most receptors can bind several ligands. It is this specificity which determines the tropism of the bacteria for a particular tissue (or a specific animal).

23.   Bacterial adherence to cells or tissue surfaces may be specific or non-specific. Non-specific adherence or "docking" involves attractive forces and allows for the approach and reversible attachment of the bacterium to the eukaryotic surface (Kachlany *et al.*, 2000). Possible interactions and forces involved include: hydrophobic interactions, electrostatic attractions, Brownian movement, recruitment and trapping by biofilm polymers interacting with the bacterial glycocalyx or capsule (Gilbert and Foley, 1997; An *et al.*, 2000; Ukuku and Fett, 2002; Foong and Dickson, 2004). Specific adherence occurs when the bacterium forms a more permanent, yet still reversible, attachment with the eukaryotic surface and may proceed as one or more steps. Many specific lock-and-key bonds between complementary molecules on each cell surface are formed. Complementary receptor and adhesin molecules must be accessible and arranged in such a way that many bonds form over the area of contact between the two cells. Once the bonds are formed, separation under physiological conditions requires significant energy input. Some Gram positive bacteria with Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMs) employ a dock, lock and latch mode of ligand binding (Ponnuraj *et al.*, 2003). Generally, reversible attachment precedes irreversible attachment but in some cases specific adherence is not observed.

24.   Mammalian cells communicate with each other through cell surface receptors. Once a receptor is bound with its ligand, a cellular response is triggered. Bacterial recognition of and interaction with host cell ligands facilitates the initial adherence to, and subsequent invasion of, host cells (Table 1). Through host receptor binding, bacteria exploit normal cellular processes to invade host cells.

25.   Many micro-organisms have elaborate properties that can be used for industrial purposes in extensive biotechnological applications. For example, *Rhodococcus* spp. have elaborated adhesive properties for attachment to environmental surfaces or for biofilm formation that are particularly useful for adherence to heavy metals and hydrocarbons (Shabtai and Fleminger, 1994; Stratton *et al.*, 2002). Although *Rhodococcus* spp. are not generally considered to be human pathogens, some species have emerged as rare opportunistic human pathogens. *Rhodococcus equi* infection is characterised by bronchiopneumonia following adherence and entry into alveolar macrophages. Garton *et al.* (2002) postulated that a novel lipoarbinomannan (LAM) variant may contribute to pathogenesis of disease caused by *R. equi.*, similar to Manosylated LAM of *Mycobacterium tuberculosis* which facilitates adherence to alveolar macrophages via mannose receptors. Evaluators must always be cognisant that those factors which have extensive industrial applications (for instance, adhesive properties) may also confer one of the properties that allow a micro-organism to cause disease in susceptible individuals.

26.   In addition to determining pathogen location, adhesins affect important aspects of the biology of infection. Many pathogens have evolved the ability to bind to cell adhesion molecules (CAMs), which are eukaryotic cell-surface receptors that facilitate cell interaction and communication with other cells and the ECM. In these cases, cell signaling processes involving actin rearrangements are affected by virtue of their contact with the cytoskeleton (Mims *et al.*, 2001). Host cell adhesion receptors can be subdivided into several groups, for example, integrins, cadherins, immunoglobulin superfamily cell adhesion molecules (IgCAMs), selectins, receptor protein tyrosine phosphatases, syndecans and hyaluronate receptors (Freemont, 1998; Hauck, 2002). Since multiple adhesion molecules are found on a single host cell, they are ideal targets for pathogens trying to anchor themselves. Often, bacteria are able to bind to cell adhesion molecules by mimicking or acting in place of host cell receptors or their ligands, and may allow bacteria to exploit several of these molecules to establish tight contact with eukaryotic cell surfaces and the ECM (Hauck, 2002; Boyle and Findlay, 2003).

Table 1.   Some examples of specific bacterial adherence to host cell surfaces

Bacterium/ Disease	Adherence Factors	Cellular Receptors	Attachment Sites	Reference(s)
Bordetella pertussis / whooping cough	fimD; FHA; pertactin; pertussis toxin	VLA5[;] β2 integrin via LRI/IAP	monocytes /macrophages respiratory epithelium	Hazenbos *et al.*, 1995; Mattoo *et al.*, 2001; Ishibashi *et al.*, 2002; McGuirk *et al.*, 2002
Burkholderia cepacia/ opportunistic infection	cable (cbl) type II pili	mucus glycoproteins	respiratory epithelium	Sajjan *et al.*, 1995
Enterococcus faecium/ opportunistic bacterimia	Collagen Adhesine Gene (*acm*)	Collagen	various tissue	Nallapareddy *et al.*, 2008
Escherichia coli – ETEC/ Diarrhoea	FaeG (F4 or K88 fimbriae, pigs); FanC (F5 or K99 fimbriae, calves, lambs); GAG (humans);	specific glycoconjugates	brush borders of intestinal enterocytes	Nagy and Fekete,1999; Van den Broeck *et al.*, 2000; Grange *et al.*, 2002
Escherichia coli - EPEC/ diarrhoea	Bfp; intimin	Phosphatidyletha nolamine; Tir	intestinal epithelium	Hicks *et al.*, 1998; Nougayrède *et al.*, 2006; Touze *et al.*, 2004
Escherichia coli - EHEC/ haemolytic uremic syndrome	intimin	Tir	colonic epithelium	Li *et al.*, 2000; Goosney *et al.*, 2001; Liu *et al.*, 2002
Escherichia coli - UPEC/ Pyelonephritis	P pili [PapG (I, II, III)]; FimH, fimbriae	Gb03, Gb04, Gb05; CD55, Gal(α1-4)Gal containing isoreceptors, mannosylated glycoproteins	kidney epithelial cells, erythrocytes; urinary tract epithelium	Dodson *et al.*, 2001; Johnson *et al.*, 2001; Ishikawa *et al.*, 2004, Nowicki *et al.*, 2002
Escherichia coli - NMEC/ Neonatal meningitis	SfaII (S fimbriae)	sialyl-α2-3 β-galactose-containing receptor molecules	endothelial and epithelial cells	Tullus *et al.*, 1992; Saren *et al.*, 1999; Bonacorsi *et al.*, 2000
Escherichia coli O157:H7	Flagellin	TLR-5	Human colonic epithelium	Miyamoto *et al.*, 2006
non-typeable *Haemophilus influenzae*/ otitis media, sinusitis, conjunctivitis	HifE (pilus adhesin); HMW1, HMW2; Hap; Hia	fibronectin; α2-3 linked sialic acid glycoprotein, unknown; fibronectin, laminin, collagen IV; unknown	respiratory epithelium	McCrea *et al.,* 1997; Laarmann *et al.*, 2002; St. Geme III 2002; O'Neill *et al.*, 2003
Haemophilus influenzae	Fimbriae LKP family	Sialic acid-containing lactosyl ceramides and AnWJ antigen	Oropharyngal epithelial cells and erythrocytes	Alphen *et al.*, 1991
Helicobacter pylori/ Peptic ulcer disease	BabA, SabAB adhesins	MUC5AC, MUC5B and MUC57	Oral cavity and stomac epithelium	Goodwin *et al.*, 2008; Lindén *et al.*, 2008
Legionella pneumophila/ Legionnaires disease	pilE; pilBCD (type IV pili); MOMP; enhD	β2 integrin (CR3), C1q, FcR	macrophage; monocytes, epithelial cells	Cirillo *et al.*, 2001; Samrakandi *et al.*, 2002

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Listeria monocytogenes/ listeriosis	InlA; InlB	E-cadherin; gC1q-R	epithelial cells	Braun *et al.*, 2000; Kathariou, 2002
Moraxella catarrhalis	UspA1 and UspA2	Fibronectin	Epithelial cells	Tan *et al.*, 2006
Mycobacterium leprae/ leprosy	PGL-1 glycolipid	α2-laminin-dystroglycan complex	Schwann cells	Marques *et al.*, 2001; Brophy, 2002
Mycobacterium tuberculosis/ tuberculosis	HbhA protein	$\alpha_M\beta_2$ integrin (CR3 or CD11b/CD18)	macrophage	Mueller-Ortiz *et al.*, 2001; Velasco-Velázquez *et al.*, 2003
Mycobacterium avium/ pulmonary disease	FAP	fibronectin	ECM (damaged epithelial cells)	Schorey *et al.*, 1996; Middleton *et al.*, 2000
Mycoplasma pneumoniae/ atypical "walking" pneumonia	P1; P30	sulfated glycolipids, sialylated compounds	respiratory epithelium, alveolar macrophages	Athamna *et al.*, 1996; Seto *et al.*, 2001; Balish *et al.*, 2003; Seto and Miyata, 2003
Mycoplasma genitalium, *M. pneumoniae*	MG: P140 and P110; MP: P1 and P30 (Specific adhesins of attachment organelles)			Burgos *et al.*, 2006
Neisseria meningitidis/ carrier state	pilC (type IV pili;) Opa; Opc	CD46; HSPGs, fibronectin, vitronectin	nasopharyngeal epithelium, endothelium	Merz and So, 2000; Dehio *et al.*, 2000; Hauck and Meyer, 2003
Porphyromonas gingivalis	Type II fimbriae	α5ß1-integrin		Nakagawa *et al.*, 2002
Pseudomonas aeruginosa	type IV pili; OprF; PA-IL, PA-IIL	asialo-GM1 - GM2; galactose- and fucose/mannose-containing glycoconjugates	epithelium	Craig *et al.*, 2004; Azghani *et al.*, 2002; Winzer *et al.*, 2000; Imberty *et al.*, 2004
Rickettsia sp.	rOmpA: Crystalline rpoteic layer (S-layer) made of Surface protein antigen (SPA)		endothelial cells	Li and Walker, 1998
Salmonella enterica serovar Typhimurium/ gastroenteritis	fim (type I fimbriae); pef (PE fimbrae); lpf (LP fimbrae); agf (curli fimbriae); ShdA	oligomannose motifs; Hep-2 domain of fibronectin	intestinal epithelium	Baumler *et al.*, 1997; Thankavel *et al.*, 1999; Kingsley *et al.*, 2004
Shigella flexneri / Dysentery	Invasion plasmid antigen BCD	$\alpha_5\beta_1$ integrin; carbohydrate moieties associated with mucin layer	colonic epithelial cells	Rajkumar *et al.*, 1998; Kerr, 1999; Kohler *et al.*, 2002
Staphylococcus aureus / boils, furuncles, impetigo, septic shock	MSCRAMMs (FnBP, Protein A, PNSG, Cna, coagulase, Clf)	$\alpha_5\beta_1$ integrin; other unknown receptors on collagen, fibrinogen, IgG, prothrombin	extracellular matrix,	Shuter *et al.*, 1996; Salyers and Whitt, 2002; Roche *et al.*, 2004
Streptococcus pneumoniae / sepsis, meningitis, otitis media, pneumonia	PavA	fibronectin	nasopharynx and alveolar epithelium	Holmes *et al.*, 2001

Streptococcus pyogenes / throat infections, other serious infections	MSCRAMMs (SfbI/F1, Fpb54, SfbII/SOF, F2)	$\alpha_5\beta_1$ integrin (fibronectin receptor)	pharyngeal epithelium	Cue *et al.*, 2000; Towers *et al.*, 2003; Kreikemeyer *et al.*, 2004
Group A Streptococcus (*S. pyogenes*)	Protein M	CD46	Keratinocytes,	Rezcallah *et al.*, 2005
Group B Streptococcus	BibA	hC4bp	Epithelial cells	Santi *et al.*, 2007
Group G Streptococcus (*S. dysgalactiae*)	Surface protein FOG	Collagens I fibrils.		Nitsche *et al.*, 2006
Treponema Pallidum / Syphilis	MSCRAMMs	fibronectin receptor containing α_5;laminin receptor	mucosal epithelium	Cameron, 2003; Lee *et al.*, 2003
Vibrio cholerae / cholera	Tcp pili; others (O Ag of LPS, MSHA, MFRHA)	specific carbohydrate and glycoprotein receptors	intestinal epithelium	Franzon *et al.*, 1993; Häse and Mekalanos, 1998; Sasmal *et al.*, 2002
Yersinia enterocolitica / diarrhoea	invasin (OMP); YadA	β_1 integrins ($\alpha_3\beta_1$, $\alpha_4\beta_1$ $\alpha_5\beta_1$ $\alpha_6\beta_1$ and $\alpha_v\beta_1$); collagen, laminin, fibronectin	intestinal epithelium and submucosa	Schulte *et al.*, 2000; El Tahir and Skurnik, 2001; Isberg and Barnes, 2001

asialo-GM1 -GM2: glycolipids
Bfp: bundle forming pili
C4bp: complement component 4 binding protein
CD46: membrane cofactor protein, member of superfamily of complement resistant proteins
CD55: decay accelerating factor for complement
Clf: clumping factor
Cna: collagen binding protein
CR1: complement receptor type 1
CR3: complement receptor type 3
Curli fimbriae: thin aggregative fimbriae
ECM: extracellular matrix
EHEC: Enterohemorrhagic *Escherichia coli*
EPEC: Entoropathogenic *Escherichia coli*
ETEC: Enterotoxigenic *Escherichia coli*
FAP: fibronectin attachment protein
FcR: Fc receptor
FHA: filamentous hemagglutinin
Fim: fimbriae
FnBP: fibronectin binding protein
FOG: Friend of GATA
HbhA: heparin-binding hemagglutinin
HSPGs: heparansulphate proteoglycans
InlA: internalin A
InlB: internalin B
LKP: long-thick fimbriae
LP fimbrae: long polar fimbriae
LPS: lipopolysaccharide
LRI/IAP: leukocyte response integrin (αVβ3, CD61) / integrin associated protein (CD47)
MFRHA: mannose fucose resistant hemagglutinin
MOMP: major outer membrane protein

MSCRAMMS: microbial surface components recognizing adhesive matrix molecules
MSHA: mannose sensitive hemagglutinin
MUC: mucin gene
NMEC: Neonatal Meningitidis *Escherichia coli*
OMP: outer membrane protein
Opa: opacity associated
Opc: class 5 outer memebrane protein
OprF: porin F
PavA: Adherence and virulence protein A
PE fimbrae: plasmid-encoded fimbriae
PGL-1: phenolic glycolipid 1
Pil: pili (fimbriae)
PNSG: Poly-n-succinyl-β-1,6 glucosamine
rOmpA: 190-kDa cell surface antigen
SfbI: streptococcal fibronectin-binding protein I
SfbII: streptococcal fibronectin-binding protein II
ShdA: host colonisation factor
Tcp: toxin co-regulated pili (demonstrably important in humans)
Tir: Translocated intimim receptor
TLR-5: Toll-like receptor 5
UspA: ubiquitous surface protein
UPEC: Uropathogenic *Escherichia coli*
VLA-5: very late antigen-5

27.   Bacterial adhesins[3] have been divided into two major groups: 1) pili (fimbriae) and 2) non-pilus (afimbrial) adhesins. Pili and fimbriae are interchangeable terms to designate short hair like structures on the surface of bacterial cells. For the purposes of this document, the terms will be used interchangeably and will depend upon the article referenced.

28.   Many bacteria express adhesive pili, which are hair like surface appendages extending out from the bacterial surface to establish contact with the surface of the host cell. Pili may be displayed circumferentially (Salyers and Whitt, 2002; Hardy *et al*., 2003) or preferentially located on one part of the bacterial cell (Nougayrède *et al*., 2006). Binding to the host cell-target is specific and it is this specificity that determines the preferential site/host for adherence.

29.   The P pilus operon serves as a useful model for the general study of different bacterial pilus systems since the concepts are similar and many of the components are interchangeable even though the host receptors differ. For example, the pyelonephritis-associated pili-D (PapD) chaperone, in addition to mediating the assembly of P pili, can modulate the assembly of type 1 pili (Bonci *et al*., 1997). There is a family of periplasmic PapD-like chaperones needed for the assembly of several pili, including K88, K99 and *Haemophilus influenzae* pili. Additionally, since the molecular machinery required for pilus biogenesis and bacterial surface assembly is conserved among diverse pili (Hultgren *et al*., 1993) the operons of type 1 and P pili are very similar with alignment of functionally analogous sequences. Nevertheless, they are structurally distinct pili (type 1 are flexible, rod-like fibers, while P pili are rigid structures) and bind to different receptors (Finlay and Falkow, 1997). Many adhesins of *E. coli* include their common pili and many strains of *E. coli* are able to express a variety of pili encoded by distinct regions of the chromosome or plasmids (Johnson, 1991).

30.   Type 1 pili produced by *E. coli* strains recognise mannose receptors on host cells (Schwan *et al*., 2002). The mannose binding site may be located at the tips or inserted along the length of the pilus. Different tip protein adhesins allow the bacterium to adhere to different host cell receptors. This is of specific interest for evaluators since changes to tip proteins can significantly alter the tropism of the bacteria for a specific receptor. For example, tip proteins on pyelonephritis-associated (pap) pili recognise a galactose-galactose disaccharide, while tip proteins on S-fimbriae recognise sialic acid. It is equally important to recognise that while a receptor may be cell- or host-specific, this specificity may also change during the developmental stages of the host. Thus, while *E. coli* has been associated with meningitis in the neonate, in the adult this association is lost. Animal studies have demonstrated that endothelial receptors for *E. coli* are only present in the brain of the newborn (Parkkinen *et al*., 1988).

31.   Type 4 pili (Tfp) constitute a separate, unique class of pili expressed by diverse Gram-negative organisms of medical, environmental and industrial importance including *Pseudomonas aeruginosa*, *Neisseria* spp., *Moraxella* spp., Enteropathogenic *E. coli* (EPEC), and *Vibrio cholera*. Tfp share structural, biochemical, antigenic and morphological features (Strom and Lory, 1993) and a biogenesis pathway that is highly conserved and resembles the type II protein secretion pathway (Wolfgang *et al*., 2000). It has been suggested that the pilin molecules located at the tip may function as adhesins since the sequences exposed differ from those packed into repeating structures within a pilus. For instance, Tfp-mediated adherence is strongly correlated with a separate tip protein, PilC for *N. gonorrhoeae,* rather than the more abundant pilin subunit protein PilE (Winther-Larsen *et al*., 2001). Alterations in the pilus subunit can also affect adherence levels. Whereas *P. aeruginosa* strains usually express only one pilus subunit, the considerable variation exhibited by this subunit by the various strains affects the proficiency of adherence of the strains.

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[3] The following paragraphs describing adhesion of bacteria to various surfaces are restricted to the pili, adhesins and secretion systems of Gram-negative bacteria.

32.   Bacteria usually adhere to receptor molecules via protein structures on their cell surface (typically pili) with distinct surface-binding capacities (Soto and Hultgren, 1999). However, other important adhesins found in a number of Gram-negative pathogens may, alternatively, be anchored directly to the outer membrane (OM), resulting in an intimate attachment with the target cell receptor (Veiga *et al*., 2003). Afimbrial adhesins are bacterial surface proteins, structurally distinct from the adhesins of fimbriae, that facilitate the tighter binding of bacteria to host cell that usually follows initial binding via fimbriae. These proteins are important components of the systems that allow bacteria to attach to and invade host cells. Some may recognise proteins on host cell surfaces while others recognise carbohydrates (Salyers and Whitt, 2002). *Legionella pneumophila* afimbrial adhesin seems to be involved in attachment to and invasion of amoebae. Adhesins require presentation on the bacterial surface in an active binding conformation for interaction with the host cell. In Gram-negative bacteria, surface localisation requires the translocation of the protein through the cytoplasmic membrane (export into the periplasm) and through the OM (secretion). Generally, surface localisation occurs via one of six different secretion pathways distinguished at least in part by the mechanisms of translocation across the OM and designated types I-VI (Stathopoulos *et al*., 2000; Cascales, 2008; Pukatzki *et al*., 2009).

33.   Proteins secreted by the type V pathway are referred to as autotransporters (AT; Henderson *et al*., 2000). For example, the *H. influenzae* Hap autotransporter is a non-pilus adhesin that influences adherence to epithelial cells and some extracellular matrix proteins and impacts bacterial aggregation and microcolony formation. Other autotransporter proteins that function as adhesins include: ShdA and MisL of *Salmonella enterica* (Kinsgley *et al*., 2002); Pertactin, Vag8 and TcfA of *Bordetella* spp. (Li *et al*., 1992; Finn and Stevenson., 1995; Finn and Amsbuagh, 1998); AIDA-I, TibA and Ag43 of *E. coli* (Benz and Schmidt, 1989; Lindenthal and Elsinghorst, 1999; Kjaergaard *et al*., 2000; Henderson and Owen, 1999); Hap, Hia and Hsf of *H. influenzae* (St. Geme III *et al*., 1994; Barenkamp and St. Geme III, 1996; St. Geme III *et al*., 1996; Yeo *et al*., 2004); BabA of *H. pylori* (Ilver *et al*., 1998); UspA2, UspA2h of *Moraxella catarhalis* (Aebi *et al*., 1998; Lafontaine *et al*., 2000) and rOmpA of *Rickettsia* spp. (Crocquet-Valdes *et al*., 1994).

34.   The AT secretion system is a modular structure consisting of three domains. These include a C-terminal transporter or β domain, an internal passenger domain and an N-terminal signal sequence. The β-domain ends up being inserted as an oligomer in the OM while the passenger domain is the protein moiety eventually presented on and anchored to the cell surface (Henderson *et al*., 1998; Veiga *et al*., 2003; Desvaux *et al*., 2004). The AT secretion system tolerate a wide range of protein modules that become displayed with the same structure, which favors the emergence of novel adhesins with new specificities. Veiga *et al*., 2003, have demonstrated this property by creating hybrid fusion proteins containing the β-AT domain of an AT protein of *Neisseria gonorrhoeae* and the partner leucine zippers of eukaryotic transcription factors Fos and Jun. When the hybrid proteins were expressed in *E. coli*, the cells acquired novel adherence traits resulting in the self-association and clumping of planktonic bacteria in liquid media, or in formation of stable consortia between cells of strains expressing the dimerisation domains.

35.   Another type of adherence is bacterial attachment to a surface and each other to form a biofilm. In a biofilm the adherence is mediated by an extracellular polysaccharide slime that acts as a kind of non-specific (although the signal to produce the biofilm may be specific) glue to bind the bacteria to each other and to a surface (Watnick and Kolter, 2000; Salyers and Whitt, 2002).

36.   Many microbes can occupy a variety of habitats, whereas others are confined to a specific microenvironment. The range of hosts, tissues or cell types colonised by bacteria is determined, in part, by adhesin recognition of and affinity for host receptors. For example, most *Bordetella* spp. can cause a similar disease in the upper respiratory tract of many mammals but their host specificities can differ considerably. *B. pertussis* is human specific while *B. bronchisepta* is responsible for infecting a wide

variety of mammals and birds but only rarely causes disease in humans. Strains of *B. parapertussis* can be divided into two groups, one which is human specific, the other ovine specific (Cummings *et al.*, 2004).

37.   While not an all-inclusive list, Table 1 gives examples of specific attachments of micro-organisms to host cell surfaces. It should be noted that many, but not all, adherence factors also play a role in invasion. For a more comprehensive review of adhesins, receptors and related structures, the reader is directed to articles by Connell *et al.*(1997), Soto and Hultgren (1999), Klemm and Schembri (2000), and Nougayrede *et al.* (2006).

2.2   Host Invasion

38.   Subsequent to attachment the bacterium may or may not invade the host, depending upon the pathogen. In any case, the host-associated pathogen must now repel the host defenses. Infection is the invasion of the host by micro-organisms, which then multiply in close association with the host's tissues. Mechanisms that enable a bacterium to invade eukaryotic cells make entry possible at mucosal surfaces. Whereas some invasive bacteria are obligate intracellular pathogens, most are facultative intracellular pathogens. In many cases, the exact bacterial surface factors that mediate invasion are not known, and multiple gene products are frequently involved. Pathogens may have mechanisms to disguise or switch antigens on their surface, thus confusing humoral and cellular immunity. Defensive mechanisms include the expression of proteins and enzymes to destroy phagocytes and weaken surrounding host tissues, making it easier to spread to new areas. Many pathogens have also developed resistance to common antibiotics, allowing them to continue infection even when the host is treated with antibiotics.

39.   Entry into tissues may take several forms. Micro-organisms may pass directly through the epithelia, especially mucous membranes that consist of a single cell layer. However in the case of skin, which is tough and multilayered, access is usually via trauma, insect bites or other damage to the surface.

40.   Invasion through mucosal surfaces requires that the bacteria first cross the mucus layer coating the epithelium and then adhere to and infect the underlying target tissue. Many micro-organisms must first interact with specific receptors on the surface of the host cell to penetrate through mucosal epithelia. Mucosal and submucosal glands secrete a protective network of carbohydrate-rich glycoproteins called mucin. Aside from the lubricative value of mucin, the primary function is to trap bacteria and prevent them from gaining access to mucosal cells. Most bacteria have mucin-binding surface molecules and are removed with the mucus flow, some establish residence within the mucus layer or penetrate the mucus and adhere to epithelial cells (Salyers and Whitt, 2002). Bacteria which lack mucin-binding surface proteins or carbohydrates may have the ability to transit the mucin layer. Since mucin is an extremely viscous material that is relatively resistant to enzymatic digestion (de Repentigny *et al.*, 2000; Moncada *et al.*, 2000) bacteria that are able to move through viscous material or degrade mucin can overcome the first major barrier to mucosal invasion. In risk/safety evaluation attention should be given, in general, to any changes in surface proteins or carbohydrate moieties involved in binding to mucin or with an ability to degrade mucin.

41.   In most cases, once a micro-organism crosses an epithelial barrier, it is recognised by macrophages (mononuclear phagocytes and neutrophils) resident in tissues. Binding to specific cell-surface receptors triggers phagocytosis. When internalised bacteria become enclosed in a membrane vesicle or phagosome, it becomes acidified by the lysosomes. Fusion with lysosomes mediates an intracellular antimicrobial response to kill the bacteria. Most bacteria are destroyed by this process, however, there are various bacterial strategies for coping with phagolysosome formation and evading destruction. One strategy prevents phagosome-lysosome fusion and is used by *Mycobacterium, Legionella* and *Chlamydia* spp. Another strategy exemplified by *Actinobacillus* spp., *Listeria* spp*., Rickettsia* spp. and *Shigella* spp. involves disruption of the vesicle membrane and entry into the cytoplasm (Gouin *et al.*,

1999). Bacterial survival and evasion of host response will be covered in more detail under the heading "2.3. Evasion of Host Immune Response and Multiplication in Host".

42.   Host invasion may be aided by the production of invasins which act against the host by breaking down primary or secondary defenses of the body. Part of the pathology of a bacterial infection may be the result of invasive activity. One of the best-studied invasins is produced by *Yersinia* spp. Isberg and Leong, (1990) demonstrated that invasin tightly adheres to β₁ integrins (host cell adhesion receptors) to mediate bacterial uptake by "zippering" the host cell membrane around the bacterium as it enters. The ability of various bacteria to induce internalisation following contact with eukaryotic cells appears to play a crucial role in pathogenesis (Finlay and Cossart, 1997). This uptake is directed into host cells that are not naturally phagocytic, including epithelial and endothelial cells lining mucosal surfaces and blood vessels, and is manipulated by the invading bacteria.

43.   The two main mechanisms of induced uptake are zipper and trigger. Bacteria utilising the zipper mechanism of entry express a surface protein which binds to host surface receptors involved in cell-matrix or cell-cell adherence. This directed contact between bacterial ligands and cellular receptors proceeds sequentially inducing host membrane extension and bacterial uptake through a "zippering" mechanism. (Cossart and Sansonetti, 2004). Various pathogens such as *Helicobacter pylori* (Kwok *et al.*, 2002), *Listeria monocytogenes* (Lecuit *et al.*, 1997), *Neisseria* spp. (McCaw *et al.*, 2004) and some streptococci (Dombek *et al.*, 1999) use this type of mechanism. With the trigger mechanism of entry, bacteria bypass the first step of adhesion and interact directly with the cellular machinery. Effectors are injected through a type III secretory system (TTSS) and the bacterial signals sent to the host cell induce prominent membrane ruffling and cytoskeletal rearrangements resulting in macropinocytosis and almost passive entry of bacteria (Finlay and Cossart, 1997). This type of system is used by *Salmonella* spp. (Hayward *et al.*, 2002) and *Shigella flexneri* (Van Der Goot *et al.*, 2004). Generally, invasion into normally non-phagocytic cells establishes a protected cellular niche for bacterial replication, survival and persistence.

44.   It must be stressed that a same single invasion strategy may not be shared by all members of a species. *Streptococcus pyogenes* strains have been shown to trigger different uptake events via distinct mechanisms. For instance, in *S. pyogenes* strain A40, the protein SfbI (Streptococcal fibronectin binding protein) has been shown to be the main factor for attachment and invasion and uptake is characterised by the lack of actin recruitment and the generation of large membrane invaginations (Molinari *et al.*, 1997). Whereas in *S. pyogenes* strain A8, the *sfbI* gene is absent and uptake involves major rearrangements of cytoskeletal proteins leading to recruitment and fusion of microvilli and the generation of cellular leaflets (Molinari *et al.*, 2000).

45.   There is little distinction between the extracellular proteins which promote bacterial invasion and various extracellular protein toxins or exotoxins which damage the host. The action of an invasin is usually proximal to the site of bacterial growth and may not kill the cells, whereas exotoxins may act at sites distant to those of bacterial growth and are usually cytotoxic. In general, exotoxins are more targeted and result in greater pathology than invasins (Henderson *et al.*, 1996; Al-Shangiti *et al.*, 2004). However, some exotoxins such as diphtheria toxin or anthrax toxin play a role in invasion while some invasins (*e.g.* staphylococcal leukocidin) have a relatively specific cytopathic effect. Table 2 lists some extracellular proteins which act as invasins. Host damage by exotoxins is more fully discussed under the heading "2.4, Ability to Damage or Kill Host".

Table 2.   Extracellular bacterial proteins that act as invasins

Invasin	Bacteria	Action	Reference
C5a peptidase	Group A and B Streptococcus	Inactivates human C5a and promotes epithelial cells invasion leading to the dissemination of bacteria	Wexler *et al.*, 1985; Cheng *et al.*, 2002
Collagenase	*Clostridium* spp.	Dissolves collagen	Borriello, 1998; Poilane *et al.*, 1998
Gingipain (cystein protease)	*Porphyromonas gingivalis*	Destruction of connective tissue, degradation of paxillin and FAK	Nakagawa *et al.*, 2006
HAD superfamily member SerB653	*Porphyromonas gingivalis*	Secreted when in contact with gingival epithelial cells	Tribble *et al.*, 2006
Hyaluronidase (see also paragraph 61)	*Streptococcus* spp., *Staphylococcus* spp. and *Clostridium* spp.	Degrades hyaluronic acid of connective tissue	Paton, *et al.*, 1993; Borriello, 1998; Hynes *et al.*, 2000
Hemolysins/ cytolysins	*Edwardsiella tarda, Escherichia coli, Bordetella pertussis, Listeria monocytogenes, Streptococcus* spp., *Staphylococcus* spp. and *Clostridium* spp..	Destroy red blood cells and other cells by lysis	Paton *et al.*, 1993; Strauss *et al.*, 1997; Bassinet *et al.*, 2000; Cockeran *et al.*, 2002; Doran *et al.*, 2002; Nizet, 2002; Seirig *et al.*, 2003
Kinases (see also paragraph 62)	*Staphylococcus* spp.. and *Streptococcus* spp.	Convert plasminogen to plasmin which digests fibrin	Ringdahl *et al.*, 1998; Gladysheva *et al.,* 2003
Lecithinases	*Clostridium perfringens, Listeria monocytogenes*		Awad *et al.*, 1995; Appelberg and Leal, 2000
Leukocidin	*Staphhylococcus aureus*	Disrupts neutrophil membranes and causes discharge of lysosymal granules	Rogolsky, 1979; Dinges *et al.*, 2000
Phospholipases	*Clostridium perfringens, Neisseria gonorrhoeae, Shigella flexneri, Pseudomonas aeruginosa*	Hydrolytic enzymes involved in phospholipid cleavage	Vasil, 1986; Awad *et al.*, 1995; Meyer *et al.*, 1997; Guhathakurta *et al.*, 1999; Edwards *et al.*, 2003
Sialidases/ neuraminidases	*Vibrio cholerae, Shigella dysentariae, Streptococcus pneumonia, Trichomonas vaginalis, Bacteroides fragilis, Gardnerella vaginalis, Mycoplasma hominis*	Degradation of sialomucin on epithelial cell layer	Paton *et al.*, 1993; Wiggins *et al.*, 2001; Stewart-Tull *et al.*, 2004

2.3   Evasion of Host Immune Response & Multiplication in Host

46.   Microbial infections rarely cause disease without first multiplying within the host. Usually, multiplication is the main cause of disease associated with bacterial infection. Following entry into a host cell, most bacteria, including pathogens, are killed by macrophages and polymorphonuclear leukocytes (PMNs). The incubation period reflects the time needed for the bacteria to overcome these early defenses and increase in number. The potential of a pathogen to cause a successful infection is reflected in the infective dose (ID). There can be wide variations in IDs, depending on the nature of the bacterial strain, the route of exposure (oral, inhalation, etc.), age (IDs would likely be lower for the very young and the very old), and the immune status of the host. Since the success of many pathogens relies on their ability to circumvent, resist or counteract host defense mechanisms, pathogens have developed numerous ways to avoid and manipulate host responses. This is reflected in the constant evolution of host defenses and bacterial pathogenic mechanisms.

47.   Phagocytes are the first line of defense encountered by bacteria following tissue invasion. Phagocytosis has two main functions: 1) disposal of microbial pathogens and 2) antigen processing and presentation for the induction of specific immune responses. Bacteria that readily attract phagocytes and are easily ingested and killed are generally unsuccessful pathogens. In contrast, most successful pathogens interfere to some extent with the activities of phagocytes or in some way avoid their attention. Bacterial pathogens have devised numerous and diverse strategies to avoid phagocytic engulfment and killing, with most strategies aimed at blocking one or more of the steps in phagocytosis, thereby halting the process. Other bacterial pathogens, exemplified by *Brucella* spp., *Mycobacterium* spp., and *Legionella* spp., survive and proliferate within "professional" phagocytes such as macrophages, neutrophils and dendritic cells. Survival inside of phagocytic cells, in either neutrophils or macrophages, protects the bacteria from antibodies, antibiotics, bacteriocides, etc. during the early stages of infection or until they develop a full complement of virulence factors.

48.   Phagocytosis comprises several steps:

- Recognition and attachment of bacteria to professional (macrophages/neutrophils) or non-professional phagocytes (*e.g.* epithelial cells). The recognition is usually receptor-mediated (*e.g.* opsonisation - Fc receptors) but can be non-specific (bulk fluid pinocytosis);

- Endocytic entry of bacteria into the phagocytic cell with the generation of a phagocytic vacuole (endosome, phagosome);

- Generation of a phagolysosome via fusion of the phagosome with primary and secondary lysosomal granules;

- Degranulation and killing through the release of lysosomal or granular contents in direct apposition to the bacteria within the phagolysosome (maybe via oxygen dependent and/or oxygen -independent mechanisms of killing).

49.   The various strategies employed by bacteria to avoid destruction by phagocytes include: 1) adaptation to withstand the antimicrobial activity of the fused phagolysosome; 2) altering phagocytosis to target the bacterium to a novel phagosome; 3) escape from the phagosome into the cytosol by lysing the vacuolar membrane; 4) blocking lysosome/phagosome fusion or attenuating the acidification of phagolysosomes; 5) circumventing or resisting phagocytosis.

2.3.1   *Adaptation to withstand the antimicrobial activity*

50.   With some intracellular bacteria, phagosome-lysosome fusion occurs, but the bacteria are resistant to inhibition and killing by the lysosomal constituents. Also, some extracellular pathogens can resist killing in phagocytes utilising similar resistance mechanisms. Resistance to phagocytic killing within the phagocytic vacuole is not completely understood but it may be due to the surface components of the bacteria or due to extracellular substances produced which interfere with the mechanisms of phagocytic killing. *Brucella abortus* and *Staphylococcus aureus* are vigorous catalase and superoxide dismutase producers, which might neutralise the toxic oxygen radicals that are generated by the NADPH-oxidase and myeloperoxidase systems in phagocytes. *S. aureus* also produces cell-bound pigments (carotenoids) that "quench" singlet oxygen produced in the phagocytic vacuole. There are some micro-organsims, however, that are dependent upon phagosome-lysosome fusion for intracellular replication and persistence. The pH that develops in the phagosome after engulfment induces bacterial gene products that are essential for their survival in macrophages. For instance, replication and synthesis of metabolic factors required for intracellular persistence of *Coxiella burnetti, Brucella suis* and *S. typhimurium* is induced by the acidic

pH found within the phagolysosome (Hackstadt and Williams, 1981; Rathman *et al*., 1996; Porte *et al*., 1999; Ghigo *et al*., 2002).

2.3.2   Alteration of phagocytosis

51.   Bacteria such as *Salmonella* spp. are able to induce phagocytosis in non-professional phagocytes. The Salmonella-containing vacuole (SCV), a unique cytoplasmic organelle formed following phagocytic induction, actually protects the bacterium; *Salmonella* spp. interfere with the ability of this phagosome to fully mature into a phagolysosome (Duclos and Desjardins, 2000).

2.3.3   Escape

52.   Escape from the phagosome is a strategy employed by the Rickettsiae. *Rickettsia* spp. enter host cells in membrane-bound vacuoles (phagosomes) but are free in the cytoplasm a short time later, perhaps in as little as 30 seconds. A bacterial enzyme, phospholipase A, may be responsible for dissolution of the phagosome membrane. *Listeria monocytogenes* relies on several molecules for early lysis of the phagosome to ensure their release into the cytoplasm. These include listeriolysin O (LLO), a cholesterol-dependent cytolysin (CDC) and two forms of phospholipase C. The low optimal pH activity of LLO allows the bacterium to escape from the phagosome into the host cytosol without damaging the plasma membrane of the infected cell.

53.   Glomski *et al*. (2002) demonstrated that a single amino acid change from leucine 461 to threonine profoundly increased the hemolytic activity of LLO at a neutral pH and promoted premature permeabilisation of the infected cells. This discovery demonstrates how minor changes in proteins can be used by bacterial pathogens to establish and maintain the integrity of their specific niches or exploited by researchers working with bacteria to produce a protein with novel properties. Once in the cytoplasm, *Listeria* spp. induce their own movement through a process of host cell actin polymerisation and formation of microfilaments within a comet-like tail. *Shigella* spp. also lyse the phagosomal vacuole and induce cytoskeletal actin polymerisation for the purpose of intracellular movement and cell-cell spread.

2.3.4   Blocking fusion or attenuating acidification

54.   Some bacteria survive inside of phagosomes by blocking the fusion of phagocytic lysosomes (granules) with the phagosome thus preventing the discharge of lysosomal contents into the phagosome environment. This strategy is employed by *Salmonella* spp., *M. tuberculosis*, *Legionella* spp. and the chlamydiae. With *Legionella* spp., it is known that a single gene is responsible for the inhibition of phagolysosomal fusion. Attenuating the acidification of phagolysosomes is observed with *Rhodococcus* spp. Toyooka *et al*. (2005) demonstrated that phagolysosomes did not acidify when they contained virulent *R. equi* organisms. Their research indicated that *R. equi* in phagolysosomes produced substance(s) to suppress acidification. Results by Tsukano *et al*. (1999) indicated that inhibition of phagosomal acidification by *Y. pseudotuberculosis* was due to attenuation of vacuolar-ATPase activity.

2.3.5   Phagocytic circumvention

55.   Bacteria may avoid phagocytosis by simply penetrating areas inaccessible to phagocytes such as the lumens of glands and the urinary bladder and surface tissues such as the skin. Other strategies for phagocyte evasion include suppression of the inflammatory response and inhibition of phagocyte chemotaxis. For example, pneumolysin (streptolysin) toxin produced by *Streptococcus pneumoniae* (Paton and Ferrante, 1983; Ernst, 2000) and components of *Mycobacterium* spp. inhibit polymorphonuclear leukocyte (PMN) migration. Also, studies involving pathogen-induced PMN alterations have suggested that *Anaplasma phagocytophilum* delays PMN apoptosis and lessens proinflammatory cytokine release (Yoshiie *et al*., 2000; Klein *et al*., 2000.). Bacteria using host cell mimicry for phagocytic evasion cover

their surface with a component which is recognised as "self" by the host phagocytes and immune system. This effectively hides the antigenic surface of the bacterial cell. Phagocytes are unable to recognise bacteria upon contact and thus opsonisation by antibodies to enhance phagocytosis is minimised. For example, *Staphylococcus aureus* produces cell-bound coagulase which clots fibrin on the bacterial surface, *Treponema pallidum* binds fibronectin to its surface, while Group A streptococci synthesise a capsule composed of hyaluronic acid which forms the ground substance of host connective tissue. Resistance to phagocytic ingestion is usually due to a component of the bacterial cell surface (cell wall, or fimbriae, or a capsule). Examples of antiphagocytic substances on the bacterial surface include: Polysaccharide capsules (*S. pneumoniae*, *Haemophilus influenzae*, *Treponema pallidum* and *Klebsiella pneumoniae*); M protein and fimbriae of Group A streptococci; Polysaccharide produced as biofilm by *Pseudomonas aeruginosa*; O polysaccharide associated with lipopolysaccharide (LPS) of *E. coli* ; K or Vi antigens (acidic polysaccharides) of *E. coli* and *Salmonella typhi,* respectively; Cell-bound or soluble Protein A produced by *Staphylococcus aureus* which attaches to the Fc region of IgG and blocks the cell-binding domain of the antibody. Whereas phagocytic resistance and intracellular proliferation is accomplished via surface components, such as bacterial capsules and LPS, which effectively shield the bacteria, resistance to many bactericidal components of host tissues is usually a function of some structural property. For example, the poly-D-glutamate capsule of *Bacillus anthracis* protects the organisms against the action of cationic proteins (defensins) or by conventional proteases in sera or in phagocytes (Fouet and Mesnage, 2002). Similarly, the OM of Gram-negative bacteria serves as a permeability barrier that is not easily traversed by hydrophobic compounds harmful to the bacteria, for example bile salts of the gastrointestinal tract. Intact LPS of Gram-negative pathogens may protect the cells from complement-mediated lysis or the action of lysozyme. The OM and capsular components of Gram-negative bacteria (*e.g. Salmonella* spp., *Yersinia* spp., *Brucella* spp., *E. coli*) can protect the peptidoglycan layer from the lytic activity of lysozyme (Hughey and Johnson, 1987; Martinez *et al.*, 1995). Mycobacteria (including *M. tuberculosis*) have waxy, hydrophobic cell wall and capsule components (mycolic acids), which are not easily attacked by lysosomal enzymes (Gao *et al.*, 2003). Other factors that enhance intracellular survival include bacterial enzymes which neutralise oxygen radicals and secreted proteolytic enzymes which degrade host lysosomal proteins. Another strategy in defense against phagocytosis is direct attack by the bacterium upon professional phagocytes. Most of these are extracellular enzymes or toxins that kill phagocytes either prior to or after ingestion and are discussed under II.4, Ability to Damage or Kill Host.

2.3.6 *Multiplication in Host*

56.   Multiplication in the host also requires that the micro-organism obtains the necessary nutrients and factors needed for growth and replication. Iron is an essential nutrient that is usually limited within eukaryotic hosts. Many pathogenic bacteria have developed regulated networks of genes important for iron uptake and storage. Also, available iron concentration may trigger the regulation of virulence gene expression (Merrell *et al.*, 2003). *Salmonella* spp. and *E. coli* produce siderophores (extracellular iron-binding compounds) which extract Fe^{3+} from lactoferrin (or transferrin) and supply iron to bacterial cells for growth. Successful intracellular lifestyle is conditional on the ability of the bacteria to obtain essential nutrients from the hostile phagosomal environment. For example, the virulence of both *M. tuberculosis* and *Salmonella enterica* (Hingley-Wilson *et al.*, 2003) is dependent upon their ability to acquire magnesium while inhabiting the phagosome.

2.4   **Ability to Damage or Kill Host**

57.   To counter infection the human host relies, initially, on the innate immune system. Prior to mounting an immune response, however, the host must first detect the pathogen. The innate immune system uses sets of recognition molecules, called pattern recognition receptors (PRRs). The Toll-like receptors (TLRs) are one of the most important pattern recognition receptor families (Armant and Fenton, 2002). PRRs bind conserved molecular structures, unique to micro-organisms, termed pathogen-associated

molecular patterns (PAMPs). PAMPs such as peptidoglycan, teichoic acids, LPS, mycolic acid, and mannose, bind to PRRs on a variety of defense cells of the body causing them to synthesise and secrete a variety of cytokines. These cytokines can, in turn promote innate immune defenses such as opsonisation, activation of proinflammatory signaling cascades, phagocytosis, activation of the complement and coagulation cascades, and apoptosis (Wilson *et al.*, 2002).

58. The host immune response plays a critical role in determining disease manifestations of chronic infections. Inadequate immune response may fail to control infection, although in other cases the specific immune response may be the cause of tissue damage and disease. Not infrequently, host defense mechanisms go overboard and it is this overaggressive immune response which contributes to the tissue damage observed with some infections. A number of bacterial proteins that act as immune modulators are presented in Table 3. This document however, will focus on specific bacterial factors directly responsible for tissue damage or host death.

Table 3. Bacterial proteins that act as immunomodulator

Bacteria/ Disease	Immunomodulator	Action	Reference
Borrelia burgdorferi/ Lyme disease	OspE	Binds factor H	McDowell *et al.*, 2004
Enterococcus faecalis	Capsular polysaccharide	Resistance to opsonophagocytic killing	Hancock and Gilmore, 2002
Francisella tularensis / Tularemia	?	Survive and multiply inside macrophages	Maier *et al.*, 2007
Streptococcus pneumoniae	PspA	Inhibitor of factor B mediating complement activation and opsonisation	Tu *et al.*, 1999
Group A Streptococcus (*S. pyogenes*)	Fba	Binds factor H and fH-like protein, contribute to phagocytosis resistance	Wei *et al.*, 2005
Streptococci	Protein M	Alteration of opsonophagocytosis by recruitment of factor H	Jarva *et al.*, 2003
Group B Streptococcus (GBS)	Capsule	Protects from opsonisation by C3. ß-protein binds factorH	Rubens *et al.*, 1987; Jarva *et al.*, 2003
Group B Streptococcus (GBS)	CspA (serine protease-like)	Evasion of opsonophagocytosis	Harris *et al.*, 2003
Lysteria monocytogenes	Lysterio-lysin O	Evasion of phagosome	Schnupf and Portnoy, 2007
Neisseria gonorrhaea	Por 1A	Binds factor H, C4 bp, mediates serum resistance	Ram *et al.*, 1998 and 2001
Neisseria meningitidis	Bind factor H	Avoids lysis by complement system	Schneider *et al.*, 2006
Staphylococcus aureus	Secretes Extracellular adherence protein (EAP)	Binds to Inter-Cellular Adhesion Molecule (ICAM)-1, fibrinogen, vitronectin resulting in the disruption of the leukocyte recruitment	Athanasopoulos *et al.*, 2006
Yersinia spp.	Yop E, T A, H and Yop J	Block phagocytosis and suppress inflammatory mediators	Fällman and Gustavsson, 2005
Yersinia enterocolica	YadA	Alteration of opsonophagocytosis by recruitement of factor H	China *et al.*, 1993

59. Bacteria produce a large number of cell-associated or secreted proteins which play a role in colonisation, infection and subsequent tissue damage. The great majority of bacterial virulence factors are secreted products that augment the survival of the bacteria and/or damage the host (Jett *et al.*, 1994;

Fournier and Philpott, 2005; Kuehn and Kesty, 2005). The following is a summary of activities of many bacterial proteins that contribute to host invasion, tissue damage or death.

60.   *Collagenase:* Collagenase, produced by *Clostridium histolyticum* and *Clostridium perfringens* (Legat *et al.*, 1994; Rood, 1998), breaks down collagen, the single most abundant protein in mammals. Collagenases are thought to play a major role in the pathology of gas gangrene caused by clostridia because they can destroy the connective tissue barriers.

61.   *Spreading Factor:* Hyaluronidase, or more descriptively "spreading factor", affects the physical properties of tissue matrices and intercellular spaces. Hyaluronidase, an enzyme produced by streptococci, staphylococci and clostridia (Bergan, 1984; Li *et al.*, 2000), is also a component of venom from snakes, spiders, jellyfish, etc (Girish *et al.*, 2004; Kuhn-Nentwig *et al.*, 2004). The enzyme attacks the ground substance of connective tissue by depolymerising hyaluronic acid thereby promoting the spread of the bacteria. Its activity also causes invasion, hence hyaluronidase is also seen as an invasin (see Table 2).

62.   *Kinases:* Streptokinase and staphylokinase are produced by streptococci and staphylococci, respectively. These enzymes convert inactive plasminogen to plasmin which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in bacterial lesions allows more rapid diffusion of the bacteria (Gladysheva, *et al.*, 2003). Like hyaluronidase, kinases also cause invasion, and are seen as invasins (see Table 2).

63.   *Sialidases/Neuraminidase:* Extracellular sialidases or neuraminidases, produced by various pathogens, have the ability to hydrolyse the sialic acid residues located on many mammalian cell membranes (Rood, 1998). The neuraminidase produced by *Mannheimia haemolytica* decreases the viscosity of respiratory mucus thus providing the bacteria with greater access to the cell surface (Zecchinon *et al.*, 2005).

64.   *Toxins:* An overview of bacterial protein toxins may be found in Alouf (2000). Many toxins act on the animal cell membrane by insertion into the membrane (forming a pore that results in cell lysis), or by enzymatic attack on phospholipids, which destabilises the membrane. They may be referred to as lecithinases or phospholipases, and if they lyse red blood cells they may be called hemolysins. Hemolysins, notably produced by staphylococci (*i.e.* alpha toxin), streptococci (*i.e.* streptolysin/pneumolysin) and various clostridia, may be channel-forming membrane toxins capable of damaging a broad range of eukaryotic cell types (Awad *et al.*, 1995; Menzies and Kourteva, 2000; Doran *et al.*, 2002). Lecithinases destroy lecithin (phosphatidylcholine) in cell membranes (Awad *et al.*, 1995; Appelberg and Leal, 2000). Phospholipases, for example alpha toxin produced by *Clostridium perfringens*, hydrolyse phospholipids in cell membranes by removal of polar head groups. Leukocidin, a bacterial exotoxin similar to streptolysin, is produced by staphylococci and specifically lyses phagocytes and their granules. Although leukocidin may be referred to as a bi-component leukotoxin (Morinaga *et al.*, 2003; Futagawa-Saito *et al.*, 2004), it should not be confused with the leukotoxins of the RTX family described below.

65.   Exotoxins have sometimes been categorised according to the cells primarily affected by the toxin. For example, leukotoxins are a group of exotoxins that produce their primary effect on leukocytes, especially polymorphonuclear cells (PMNs). *Mannheimia* (*Pasteurella*) *haemolytica*, one of the key pathogens associated with bovine respiratory disease complex produces a leukotoxin (LKT) that both activates and kills bovine leukocytes. Atapattu and Czuprynski (2005) have shown that LKT produced by *Mannheimia haemolytica* induces apoptosis of bovine lymphoblastoid cells (BL-3) via a caspase-9-dependent mitochondrial pathway. While LKT is able to bind leukocytes from various animal species, it is only cytotoxic for ruminant leukoctyes. This virulence factor is a member of the RTX (repeats in toxins) family of multidomain Gram-negative bacterial toxins. RTX toxins fall into two categories: hemolysins

which attack different cell types from a variety of species and leukotoxins which show a marked specificity for both cell type and host species (Lally *et al.,* 1999). Other bacteria that produce RTX toxins include: *E. coli* (hemolysins), *Bordetella pertussis, Actinobacillus* spp. and *Actinobacillus actinomycetemcomitans* and various *Pasturella* spp. (leukotoxins), (Narayanan *et al*., 2002; Davies *et al*., 2002; Ward *et al*., 2002).

66.   *Toxins With Short-Range Effects Related to Invasion:* Bacterial protein toxins which have adenylate cyclase activity, are thought to have immediate effects on host cells that promote bacterial invasion. One component of the anthrax toxin (EF or Edema Factor) is an adenylate cyclase that acts on nearby cells to cause increased levels of cyclic AMP and disruption of cell permeability (Leppla, 1982). One of the toxin components of *Bordetella pertussis,* pertussis adenylate cyclase, has a similar effect. These toxins may contribute to invasion through their effects on macrophages or lymphocytes in the vicinity which are playing an essential role to contain the infection.

67.   *Coordination of expression of virulence factors; quorum sensing (QS).* To establish an infection, bacteria carefully orchestrate a number of bacterial factors and determinants which have a role in determining pathogenicity. Proficient coordination of these factors is required for bacterial survival and successful colonisation. Thus, bacteria have developed sophisticated regulatory systems to adapt gene expression to changing environmental conditions. The notion that bacteria can signal each other and coordinate their assault patterns against susceptible hosts is now well established (Miller and Bassler, 2001). When invading their host, bacteria do not operate in isolation. Pathogens employ a series of chemical signals and sensing systems that jointly engage bacterial communities to genetically respond in concert to specific conditions in the host and promote an advantageous lifestyle within a given environmental niche. A central component in this process is a sophisticated communication system known as quorum sensing (QS) (Ng and Bassler, 2009). QS systems regulate microbial pathogenesis through the following points: (1) helping pathogens invasion and colonization; (2) regulating production of virulent factor; (3) giving pathogens the ability of immunity or drug resistance (Wu and Xie., 2009).

68.   QS was first observed in the marine halophilic bioluminescent bacterium *Vibrio fischeri* (Nealson *et al.*, 1970), in which the bacterial light-emitting luciferase operon is activated when the population reaches a threshold concentration. It was later realized that QS is achieved through the production, release, and subsequent detection of, and response to threshold concentrations of signal molecules called autoinducers, which are synthesized throughout the growth of the bacterium. When a threshold concentration is reached, these signals interact with a transcriptional regulator, allowing the expression of specific genes (Bassler, 2002).

69.   QS systems were shown to regulate a multitude of transcriptional programs in bacteria *in vitro* and probably *in vivo*, which are relevant for the pathogenic phenotype. These include biofilm formation, growth potential, antibiotic resistance expression and genetic determinants of virulence (Kendall and Sperandio, 2007; Yarwood and Schlievert, 2003; Mack *et al*., 2007; Kong *et al*., 2006; Costerton *et al*., 2003; Bjarnsholt *et al*., 2010). That QS has a fundamental role in bacterial pathogenesis was confirmed as researchers began to find that many clinically relevant microbial pathogens, displayed auto-inducer systems homologous to the one discovered in *V.fischeri*. Many common bacterial pathogens, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacteroides*, *Yersinia*, *Burkholderia* and *Enterococcus* spp., and many clinically important staphylococcal and streptococcal pathogens were shown to contain QS genes, which participate in the regulation of multiple bacterial genes, including virulence genes (Miller and Bassler, 2001; Greenberg, 2003; Cámara *et al*., 2002; Shiner *et al*., 2005; Qazi *et al*., 2006; Parsek and Greenberg, 2000; Brady *et al*., 2008; Williams, 2007).

70.   QS circuits can also regulate human transcriptional programs to the advantage of the pathogen. Human stress hormones and cytokines can be detected by bacterial quorum sensing systems. By this

mechanism, the pathogen can detect the physiologically stressed host, providing an opportunity to invade when the patient is most vulnerable. (Li *et al.*, 2009).

71.   QS systems are broadly grouped into three categories. The quorum sensing systems identified in many Gram-negative bacteria mostly resemble the typical quorum sensing circuit of the bioluminescent bacterium *V. fischeri* (Miller and Bassler, 2001; Smith *et al.*, 2006) in which they consist, at a minimum, of homologues of the two *V. fischeri* regulatory proteins called LuxI and LuxR. The LuxI-like proteins (the auto-inducer synthases) are responsible for the biosynthesis of a specific acylated homoserine lactone (AHL) signaling molecule, termed type 1 autoinducers (AI-1). The autoinducer concentration increases with increasing cell-population density. The LuxR-like proteins (the transcription factors) bind cognate AI-1 autoinducers that have achieved a critical threshold concentration and the LuxR-autoinducer complexes activate target gene transcription, including virulence genes (Wagner *et al.*, 2007). Over 50 species of gram-negative bacteria produce AHLs that differ only in the acyl side chain moiety, and each LuxR-type protein is highly selective for its cognate autoinducer signal molecule (Bassler, 2002).

72.   The autoinducers in the QS system of a gram-positive bacterium are short, usually modified peptides processed from precursors. In contrast with the diffusible behaviour of AI-1 autoinducers, these signals are actively exported out of the cell (through an ATP binding cassette transporter, ABC-transporter), and interact with the external domains of membrane bound sensor proteins. Signal transduction triggers a phosphorylation cascade that culminates in the activation of a DNA binding protein that controls transcription of target genes. Similar to gram-negative bacteria, gram-positive bacteria can use multiple autoinducers and sensors. (Bassler, 2002).

73.   Finally, a third QS pathway, initially discovered in the *V. harveyi* bioluminescence system, is mediated by the *luxS* gene locus (the autoinducer synthase gene) and related homologues. Signaling elements in this system, termed type 2 autoinducers (AI-2), are composed of rather complex, unusual, multiple-ringed, cyclical furanosyl-borate diester molecules. The AI-2 pathway uses a more complex, two-component membrane receptor, LuxPQ, comprised of periplasmic binding protein (LuxP) and histidine sensor kinase (LuxQ) subunits (Neiditch *et al.*, 2006).Components of this system are detectable in almost one-half of all sequenced bacterial genomes, so this system is now recognized as the most ubiquitous signaling system employed by both Gram-negative and Gram-positive bacteria. It has been proposed that the AI-2 pathway is a more universal, interspecies chemical language (Bassler, 2002).

74.   Quorum sensing molecules and systems show a remarkable array of very complex properties. These systems are also capable to influence environmental processes. Geochemical and biological modifications of signals probably occur in extracellular environments, and these could disrupt or interfere with intended communication signals. It has been postulated that quorum sensing occurs within cell clusters, where signal dispersion might be significantly influenced by existing extracellular polymers (Decho, AW *et al.*, 2010).

SECTION III - MOLECULAR ASPECTS OF PATHOGENICITY

3.1 Molecular Genetic Definition of Bacterial Virulence

75.   The application of molecular biology to microbial pathogenesis was described by Falkow (1988) 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. Meeting these postulates requires the technical possibility to directly affect the genes in question, and, even more important, the availability of models to measure virulence. As this is not always feasible, an alternative approach was added: (iv) the induction of specific antibodies to a defined gene product should neutralise pathogenicity. This fourth postulate 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.

76.   Like Koch's postulates, the 'molecular Koch's postulates' cannot always be applied rigidly. If the virulence phenotype is multifactorial, as will usually be the case, the gene products identified as virulence factors may either be a 'classical' virulence factor, or an accessory factor that is essential for expression of the phenotype, but not directly involved in it. As an example: the fimbriae, hair like surface structures, that are virulence factors of uropathogenic *Escherichia coli* strains carry an adhesin molecule at their tip that performs the directly virulence related task of adherence to epithelial cells of the host. They can however only efficiently perform this task when carried at the tip of the fimbriae that are composed of other protein molecules that lack the adhesive property. The gene identified as a virulence factor may not even be a structural gene, coding for a gene product, but may have a regulatory function in the expression of the structural gene. In the literature there is a tendency to describe all genes that pass the tests described in the molecular Koch's postulates as virulence genes. This approach has resulted in the identification as 'virulence genes', as genes that are not directly involved in virulence as such, but are indispensable for the expression of the virulent phenotype because they are required in some way for correct expression of virulence genes. In fact, the molecular approach may detect a whole spectrum of 'virulence genes' ranging from 'true' virulence genes to genes encoding 'house-keeping enzymes' that through some remote mechanism influence the virulence phenotype. This may indicate a need for a more restrictive definition of virulence genes than simply genes that are detected in virulence assays.

77.   A definition of bacterial virulence should enable the discrimination between 'true' virulence genes that are directly associated with the virulent phenotype, and accessory genes, that will also be identified as virulence genes by screening methodologies that rely on gene inactivation resulting in attenuation of virulence. A well-known example of a housekeeping gene identified as a virulence factor is the gene *aroA* (as well as other 'housekeeping' genes, see Uzzau *et al.*, 2005), inactivation of which results in attenuation of pathogenicity. The *aroA* gene, however, is involved in aromatic amino acid biosynthesis, and as such is present in both pathogens and non-pathogens and is not considered a virulence gene. This is easily understood in the case of *aroA,* but when the gene product has no known housekeeping function, such genes would be described in the literature as virulence genes. The problem is where to draw the line in the continuum between 'virulence-associated genes', and 'housekeeping genes'. 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. However, this would exclude certain well-characterised virulence genes, for instance lipopolysaccharide (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 cannot be sharply defined.

3.1.1   *Molecular approaches to identify virulence genes*

78.   Three basic approaches are used to identify virulence genes: genetic methods to obtain phenotypic evidence for virulence, methods based on the proposed immunogenicity of virulence factors for immunological evidence, and comparative genetic methods that yield additional indirect evidence.

79.   *Phenotypic evidence.* Within the genetic methods two approaches are used: (i) inactivation of a virulence gene must result in loss of virulence; or (ii) introduction of a virulence gene into a non-virulent strain must add virulent properties. It should be noted that both principles are heavily dependent on models to determine the virulent phenotype. Models to determine virulence are ideally animal models that minutely mimic natural disease, but these are not always available. More often animal models have to be 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. The complicated interaction of host and bacteria is often ignored when *in vitro* models are applied. 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 complementarity. Inactivation of one link of the chain may eliminate invasiveness, but complementation in a different setting may require several genetic loci. 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 alternatives.

80.   *Immunological evidence.* A second approach for identifying virulence genes is based on the proposed immunogenicity of virulence factors. Knowing that acquired immunity can protect against disease, it is assumed that protective antibodies are directed against virulence-associated genes. When an infection results in an antigenic response directed against one or more specific antigens, this is taken as a strong indication that these antigens represent virulence-associated factors.

81.   *Comparative genetical evidence.* Examples of the genetic approach to identification of potential virulence-associated genes are the identification of (i) genes with a degree of homology to known virulence-associated genes that is considered significant in bioinformatics surveys; (ii) related genes that show variation that can be interpreted as antigenic variation; (iii) genes that are shown to be present in (more) virulent strains, while absent in avirulent strains. Comparative genetic approaches are further discussed in section 3.1.2, on trends in virulence gene identification.

82.   In addition to these approaches, several techniques have been developed to identify and characterise bacterial genes that are induced during the intracellular infection and therefore, potentially, may play a role in pathogenesis. Examples are the search for genes that are specifically induced in the host, and 'signature-tagged mutagenesis' (STM), involving comparative hybridisation to isolate mutants unable to survive the environmental conditions in the host (Mahan *et al.*, 1993; Chiang *et al.*, 1999; Harb and Abu Kwaik, 1999). A very powerful approach to isolate mutants that may be affected in a virulence gene is STM as discussed by Autret and Charbit (2005). The general technique of STM can be applied to find specific genes involved in survival persistence of a bacterium in a host; virulence genes would fall into this class of genes (Wassenaar and Gaastra, 2001). The only prerequisite for a gene to be found by STM is that

its loss of function should not result in a lethal phenotype under conditions of growth *in vitro*, in broth. This is probably not an impediment for most virulence genes to be identified by this technique. The STM approach involves transposon (usually) mutagenesis of a bacterial strain, followed by pooling of a number of mutants that can be individually recognised by a polymerase chain reaction (PCR) amplifiable tag. The pooled mutants are inoculated in an animal model, and bacteria retrieved from the animal are analysed for mutants that are present, as shown by the presence of their tag. Mutants that are lost have been mutated in genes that have a function in the pathogenic process, or which at least have a function is needed to survive and be retrieved in the experiment.

83.   Ideally, for the identification of virulence, several approaches should lead to the same gene or set of genes, and the characterisation of a gene as virulence-associated should be based on evidence from more than one approach. Even then, the controversy between housekeeping genes and virulence genes is not always solved. For example, the housekeeping magnesium transport system of *Salmonella typhimurium, mgtA/B*, is under *PhoP/PhoQ* regulation, and is activated during invasion *in vitro* (Smith and Maguire, 1998). One 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 (Klose and Mekalanos, 1997). 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 colonisation. Since glutamine synthetase is also present in non-pathogenic bacteria it is not considered a virulence gene in the comparative genetic approach. As the absence of virulence genes in non-pathogenic bacteria receives a lot of weight in this approach, two points need to be considered: 1) the outcome of such comparative genetics is heavily dependent on the content of the databases used; and 2) gene function is not always correctly predicted by comparative genetics alone. Putative virulence gene candidates identified in this way should therefore at least be confirmed by phenotypic evidence, despite the mentioned shortcomings of such evidence.

3.1.2   *Trends in virulence gene identification*

84.   Due to explosive developments in genomics it is now feasible to analyse the complete genome of bacterial pathogens by *in silicio* subtractive hybridisation. With the expanding annotation of genes from genome sequences, this can lead to the identification of new virulence genes (Field *et al*., 1999; Frosch *et al.,* 1998). The annotation of these newly sequenced genes is based on sequence identity. This identification of virulence genes by comparative genomics, based on genetic similarity is, however, risky for several reasons.

85.   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-characterised analogue in another species. However, genes may have a niche-adapted function in a particular organism, and this may have its effect on the role of the gene product in virulence. Functional domains may not be conserved (Lee and Klevit, 2000). Therefore, sequence homology does not always predict function, and even when there is a high degree of genetic conservation between a non characterised gene and a known virulence gene, the function of the gene product of the non characterised gene as a virulence factor should first be experimentally tested before functional homology is assigned. Until then, the newly identified virulence gene should be annotated as 'putative'. 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.

86.   Another, diametrically opposed, pitfall of comparative genetics is that genes that share no sequence homology can have identical functions, as is demonstrated for *actA* of *Listeria monocytogenes*

and *IcsA* in *Shigella flexneri* whose gene products recruit host cell actin (Strauss and Falkow, 1997). This type of functional similarity will go unnoticed by genome comparison.

87.   Many virulence genes display antigenic polymorphisms, presumably to evade the selection pressure of the host immune system (Deitsch *et al*., 1997). The correlation between polymorphism and virulence is so strong that polymorphisms observed *in silicio* are taken as indirect evidence for a role in virulence. It should be noted that the term polymorphism is used for different phenomena. 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. 'Polymorphism' is also 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 (Van Belkum *et al*., 1998). 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.

88.   In conclusion, different paths lead to the identification of virulence genes. A 'top down' approach, starting from a single pathogen, will start by defining the pathogenic phenotype of the organism ('adhering', 'invasive', 'toxin producing', 'phagocytic survival'), and subsequently zoom in on the virulence genes responsible for this phenotype. A 'bottom up' approach will start from an annotated genome sequence, from which putative virulence genes can be identified by comparative genetics. The relevance of such identified putative virulence genes for the pathogenic phenotype then remains to be proven. For this a 'lateral' approach can be useful, as pathogens often employ similar pathogenic mechanisms, and analogies between virulence factors can be used 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. Section II presents an overview of genes that are involved in different stages of pathogenicity: host recognition and adherence, host invasion, multiplication in the host, the ability to overcome the host immune response and host defense systems and the ability to damage or kill the host.

3.1.3   *The perspective of virulence genes*

89.   Understanding of bacterial virulence factors can be biased because of the experimental setup applied to identify or study the factor (Quinn *et al*., 1997). For instance, many bacterial toxins are described as 'haemolysin', because they have been originally recognised as cytolytic to erythrocytes. However, in real life these toxins may not be targeted at erythrocytes, but at leukocytes or other host cells instead. This is just one example of how the perception of bacterial virulence factors is influenced by experimental design.

90.   Pathogenicity and virulence are often addressed in an anthropomorphic manner, *i.e.* the incorrect concept that it is the 'aim' of pathogenic bacteria 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. Examples are the release 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, and may, for instance, be dependent on the immune response of the individual (Casadevall and Pirofski, 1999). Conditions that result in disease can vary among host individuals, and from host species to host species. This adds to the difficulties to identify the bacterial genes that are directly responsible for the disease. Ideally, experimental shortcomings,

subjective observations, and the anthropomorphic view on pathogenicity should all be considered when establishing the relevance of a certain virulence gene to the pathogenicity of a micro-organism.

3.1.4   *Classification of virulence genes*

91.   From the previous sections it will be clear that there are many ways of defining, identifying, and testing virulence genes. But, since each pathogen has evolved to fit its own niche, different pathogens do not necessarily share common pathogenic characteristics. Despite the recognition of common themes in bacterial virulence (Finlay and Falkow, 1989, 1997), 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.

92.   As already stated in the introduction (section I), regulators dealing with risk/safety assessment of genetically engineered bacteria need a good understanding of the significance of a given virulence gene in its physiological background; only if the newly acquired gene can have a role in the pathogenic life style of the recipient micro-organism can an interaction be expected between the newly acquired gene and the resident genes contributing to the pathogenic lifestyle. Wassenaar and Gaastra (2001) have proposed a classification of virulence genes according to their potential role in pathogenic lifestyles that should be helpful to evaluate the potential influence of newly acquired genes on virulence.

93.   Wassenaar and Gaastra (2001) discriminate among four lifestyles: exclusive pathogens, host dependent pathogens, opportunistic pathogens and fully non-pathogenic organisms. Seven types of virulence gene classes are distinguished: true virulence genes, directly involved in interactions with the host and responsible for pathological damage (*e.g.* toxins); colonisation genes, determining the localisation of the infection; host defense system evasion genes (*e.g.* immunoglobulin specific proteases); processing genes involved in the biosynthesis of virulence life style factors (*e.g.* chaperonins; gene products with a virulence life style substrate), secretory genes, virulence housekeeping genes (*e.g.* urease, catalase), and regulatory genes, involved in the regulation of virulence life-style genes. Further subclasses may be identified for these classes.

3.2   **Evolution and Spread of Virulence Genes; Pathogenicity Islands**

94.   In general, three mechanisms can be proposed for the evolution of pathogens: acquisition of virulence genes from existing pathogens by horizontal gene transfer; a change in host specificity (host jump) of an existing pathogen, possibly together with, or as a result of, the acquirement of genes to adapt to a new ecological niche; and evolution of new virulence genes from the existing gene pool of a bacterial species, resulting in (an increase of) virulence.

95.   Over the past few years it has become apparent that of these, the evolutionary consequences of horizontal gene transfer are probably the most drastic. There is ample evidence that virulence genes have spread by horizontal gene tranfer, by all processes known to contribute to the process [see the OECD guidance document on horizontal gene transfer between bacteria (OECD, 2010)]. Of special importance are bacteriophages, that confer virulence factors to bacteria (Boyd and Brüssow, 2002; Wagner and Waldor, 2002).

96.   In the late 1980s Hacker and colleagues (Dobrindt *et al.*, 2004, see also Schmidt and Hensel, 2004) were the first to notice that pathogenicity related genes are often located on mobile genetic elements, called 'pathogenicity islands' (PAIs). PAIs may be identified as strain specific sequences by subtractive hybridisation between virulent and avirulent strains of the same species. They are frequently found integrated in or near to tRNA genes, which have perfect properties for docking sites because they are

highly conserved and often present in multiple copies. They are characterised by (the remains of) insertion sequences (IS) at their borders that, if still functional, may lead to genetic instability and to spread of the PAI to other strains by horizontal gene transfer. The GC content and codon usage of PAIs is often different from the GC content and codon usage of the rest of the genome, which is taken as an indication of their recent acquisition in the genome.

97.   PAIs typically contain sequences that code for gene products that have a (putative) role in virulence. The uropathogenic strain 536 of *E. coli* that has been extensively studied by the group of Hacker (Brzuszkiewicz *et al*., 2006), provides a good example of what might be found on PAIs. *E. coli* 536 contains seven PAIs coding for different types of fimbriae, haemolysins, a capsule, a siderophore system, a Yersiniabactin, proteins involved in intracellular multiplication, and for a hybrid peptide-polyketide genotoxin that causes cell cycle arrest and eventually cell death of eukaryotic cells that are in contact with this *E. coli* strain (Nougayrède *et al*., 2006).

98.   The ongoing elucidation and analysis of prokaryotic genomes has shown that not only pathogenicity related traits are located on 'islands'. PAIs are a specific example of a 'genomic island' (GEI), the term that has been coined for the phenomenon that bacteria carry in their genome a flexible gene pool that encodes additional traits that can be beneficial under certain circumstances, and that allows them to occupy a specific niche, while the more constant part of the genome takes care of 'household' functions. GEIs are commonly found in the 'metagenome', *i.e.* the combined genomes, of bacteria that share a niche. They would appear to facilitate exchange of useful genes between these bacteria that are mutually supportive in occupying the same environment. The traditional view of bacterial evolution occurring through clonal divergence and selection must be broadened to include gene exchange as a major driving force for adaptation to specific niches. PAIs would be an example of this phenomenon of gene transfer, in facilitating bacteria to function as pathogenic organisms.

99.   The genome flexibility that leads to enhanced virulence is not restricted to acquisition of virulence factors; it may also include loss of genomic sequences, as has been shown for *Shigella flexneri* and enteroinvasive *E. coli* (Maurelli *et al*., 1998). In general, it appears to be evolutionarily profitable to counteract the acquisition of genes that provide selective advantage with loss of genetic information that can be dispensed with in the new niche, as is the case for instance for intracellular symbionts.

100.   In conclusion pathogenicity is not a singular trait of a singular type of organism, 'the pathogens'. Rather, pathogenic traits are adaptive traits that equip a bacterium for a specific lifestyle in a specific niche that happens to be the surface or interior of the host's body.

SECTION IV - ASSESSING POTENTIAL FOR BACTERIA-MEDIATED ADVERSE HUMAN HEALTH EFFECTS

101.   In the previous sections it has been argued that deleterious effects that are caused by pathogenic organisms can be understood as effects of a 'life style', or constellation of traits, that enables these organisms to colonise and use specific environments in or on the human or, in general, animal body as a niche. This line of thinking has been broadened by Casadevall (2006), who has pointed out that many micro-organisms in the environment have developed lifestyles that allow them to interact with the other organisms that they encounter in the environment. The same or similar gene products may have a role in different lifestyles, *e.g.* the interaction with fungi, protozoa as well as vertebrates. Bacterial strains that have no directly apparent role in human pathogenicity, for instance because they do not survive or replicate at 37° C, may still carry genes that code for gene products with a potential role as virulence factors in bacteria that are more compatible with a lifestyle as a human pathogen. DNA exchange between microbial strains may in the end provide bacteria that thrive in the human environment with new virulence factors derived from such bacteria. This complicates the risk/safety assessment of deliberate release of environmental strains, particularly if these strains have been subject to genetic engineering. The following is intended to help the assessor negotiate these complications.

4.1   Risk/safety assessment of environmental release of bacterial strains to determine whether these may cause adverse human health effects

102.   Environmental release of bacteria should be preceded by a risk/safety assessment. Risk assessment usually comprises four steps: 1) hazard identification; 2) hazard characterisation (*e.g.* dose-response assessment); 3) exposure assessment (dose, concentration, survival); and 4) risk characterisation. In the risk assessment of environmental releases of bacterial strains, one aspect that has to be taken into account in hazard identification and hazard characterisation is the pathogenic potential of the bacteria to cause adverse human health effects.[4] The WHO Laboratory Biosafety Manual [WHO (2004), chapters 2 and 16] provides helpful considerations on the risk/safety assessment of (potentially) pathogenic organisms. These considerations apply primarily to laboratory settings, but they can be easily adapted and applied to environmental settings.

103.   For genetically engineered bacteria, the risk group (see section 1.2) of the species is a first approximation of the degree of bacterial pathogenicity in humans. But assessing the degree of pathogenicity of a bacterial strain calls for an unequivocal identification of the location of the strain in the spectrum from clear non-pathogen to clear pathogen. This should be done with caution. Truly non-pathogenic bacteria will lack the ability to survive in a human host (with the exception of commensal bacteria), or cause any adverse effects. Bacteria that are incompatible with the human environment *e.g.* bacteria that cannot survive at temperatures between 30-42° C, or that are exclusively phototrophic or lithotrophic would be expected to be non-pathogenic. Still, one should be careful drawing this conclusion.

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[4] Other aspects that have to be taken into account in various steps of the risk assessment include (but are not limited to) natural background levels; conditions for survival, persistence, growth, and reproduction; mode of dispersal; potential for gene transfers, in particular genes associated with pathogenicity, toxicity or persistence; antibiotic resistance.

For instance, lithotrophic bacteria have been found in infections associated with surgical implants in the human body (Dempsey *et al.*, 2007). Indeed, Casadevall (2006) has pointed out that bacterial strains that have lifestyles that do not link them to pathogenicity in humans can carry genes that code for gene products with a potential role as virulence factors in bacteria that are or could develop into human pathogens. Falkow (2008) argues that it is difficult to separate the pathogenic from the commensal lifestyle. What is the difference between a pathogen and a commensal? Pathogens possess the *inherent* ability to cross anatomic barriers or breach other host defenses that limit the survival or replication of other microbes and commensals. Therefore, most, but certainly not all, pathogens establish themselves in an environment usually devoid of other stable microbial populations. These invasive properties are essential for their survival in nature, and, are often host specific. However, many "commensal" bacteria, that are able to colonise the human host without displaying immediate virulence phenotypes, can cause disease, (*e.g.* Group A and B streptococci, *S. aureus, N. meningitidis, S. pneumonia, H. influenza*). Many features that are seen as virulence factors of pathogens (pili, antiphagocytic proteins, capsules) may also be found in non-pathogenic bacteria. Virulence factors may simply be examples of a more general class of "adaptive factors" common to all bacteria (Casadevall, 2006).

104.   Bacterial strains that have been derived from wild type isolates from the environment may have changed significantly in their pathogenicity compared to fresh wild type isolates. This may in particular be the case if the strains have been mutated, *e.g.* for attenuation, or if new virulence related genes have been introduced by genetic engineering, have been lost afterwards. But also the fact that strains have been handled in the laboratory during many generations, may have led to the occurrence of mutants with changed properties. Losses of properties that have no function in survival under laboratory conditions, like virulence factors, occur quite frequently under these circumstances. As in all cases of attenuation, strains that have been attenuated in this way should be thoroughly tested for stability of the non-pathogenic phenotype (see also paragraph 12).

105.   Predicting the effects of introduced (potential) virulence genes on the pathogenicity of the recipient strain is not straightforward[5] (see section 4.2). If 'true virulence genes' (as defined in section 3.1.4) have been introduced, an effect on pathogenicity is more likely, as these genes are directly responsible for pathological damage, such as toxins. However, the degree of damage that these gene products can cause is highly dependent on the context of the pathogenic 'lifestyle' of the bacterium, which depends on the secondary virulence factors available in the bacterial strain. This discussion is especially pertinent for medical and veterinary applications of potentially (non)pathogenic strains. The discussion is also highly relevant for risk/safety assessments of releases of environmental strains that have been engineered in the laboratory. The case of the *Burkholderia cepacia* complex (Bcc) is an example of a group of host species where the effect on pathogenicity of introduced potential virulence genes could be difficult to predict. It has has already been mentioned that these bacteria occur normally in the environment, but are now recognized as an important human health hazard. (see paragraph 9). The species in this complex have for a long time been seen as (potential) plant pathogens, and their potential as human (opportunistic) pathogens has only recently been recognised. It would require more insight in the lifestyle of these bacteria to be able to predict the role of introduced virulence genes in the establishment of pathogenic potential. In cases like this, caution should be used in establishing conclusions on their pathogenicity.

106.   The likelihood of the strain actually causing adverse human health effects will depend on the exposure of humans to the bacterial strain during and after the release. Adverse effects are only to be expected if the exposure is such that it will lead to contact of humans with the bacteria in sufficient

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[5] One factor that complicates this prediction is the influence of the condition of the host, *e.g.*, the immune status of the host, on the effectiveness of a virulence factor. In regulatory discussions, however, this complication is usually evaded, as for the outcome of the discussion the conditions of the host is usually supposed to be 'normal'.

numbers in relation to the infectious dose of the specific bacterium, and in such a way that pathogenic effects may ensue, *e.g.* by ingestion, inhalation, dermal contact. Factors that determine the degree of exposure are: a) the number of bacteria released into the environment, b) physiological state of the bacteria, *e.g.* due to fermentations conditions prior to the administration, c) spread during release, dependent on the method used, *e.g.* by aerosols, injection or mixing in the soil, seed coating, d) survival, and e) dissemination after release, *e.g.* through surface and subsurface water movement, by soil fauna, or by disturbance of the site of application. Spreading of aerosols is dependent on conditions of wind at the time of the application; survival of bacteria in aerosol droplets is dependent on environmental factors, *e.g.* temperature, humidity and UV radiation. Survival of bacteria in soil is variable for different strains of the same species. In many cases where bacteria have been introduced into the environment a rapid decrease has been observed, *i.e.* the number of detectable bacteria drops below the detection limit[6] of a direct viable count within months or even weeks. This is even the case for many strains that are well-adapted to a soil-lifestyle, *e.g.* the root colonising *Pseudomonas* spp. (Glandorf *et al.*, 2001; Weller, 2007). The bacteria are however not 'lost' from the environment, and may appear again readily if environmental circumstances are favorable, for instance if the plant that they are prone to colonise is again present in the environment; also in some cases long-term survival and persistence of introduced microorganisms has been demonstrated (Hirsch, 1996).

107. If a human health hazard is expected, risk estimations should be made based on worst case assumptions on survival and spreading. Risk estimates may be refined if the results of further research show that the worst case assumptions are not realistic.

4.2 General considerations for assessing altered pathogenicity of micro-organisms as a result of genetic engineering

108. The risk/safety assessment of genetically engineered micro-organisms requires careful consideration of numerous factors, not the least of which is the genetic composition of both the recipient and the donor organisms, and their respective lifestyles and phenotypic expression. While the intended use of the organism is factored into the initial assessment, some foresight should be given to potential unintended uses, in particular if the genetically engineered strains are meant to be commercially available.

109. Genetic engineering may cause, advertently or inadvertently, changes in the various factors that determine the niche of a bacterium, and may broaden its niche, that then needs to be redefined. As described in the previous sections, pathogenicity is the capacity to cause disease, and is related to the ability of a micro-organism to reach and occupy a particular habitat on or in the host and to subsequently cause harm to the host. Thus, when performing an assessment of pathogenic potential to humans, one should consider how the engineering may change a bacterium's capacity to cause disease.

110. There are several determinants that should be considered when assessing the potential for bacterial pathogenicity as a result of genetic engineering. Consideration should be given to the biological and ecological characteristics of the non-modified strain, *i.e.* its 'lifestyle', in as far as it is compatible with causing pathogenicity in humans. Due to the lifestyle of the vast majority of bacterial strains in the environment, *e.g.* psychrophilic or thermophillic, lithotrophic or phototrophic bacteria, it is not likely that they will turn into potential human pathogens just by the introduction of one virulence factor derived from a human pathogen. On the other hand, genes derived from bacteria that are not suspected human pathogens on the basis of their lifestyle may still code for gene products that can contribute to future virulence (Casadevall, 2006).

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[6] For guidance on the detection of bacteria in soil, see the Guidance document on methods for detection of micro-organisms introduced into the environment: bacteria (ENV/JM/MONO(2004)7), http://www.rebeca-net.de/downloads/oecd%20guid%20doc%20detection%20of%20micro-organisms.pdf

111.   Genetic engineering may involve genes whose products are not inherently harmful but adverse effects may still arise from the modification or exacerbation of an existing constellation of traits in the recipient microorganism. This may arise as the result for example of the product of an inserted gene acting alongside existing pathogenic determinants or the addition of a trait completing the suite of traits necessary for a pathogenic lifestyle. Alternatively it is possible that modification of normal genes may also alter pathogenicity. In an assessment for environmental risk/safety of an application intended for environmental release, the following points should be considered:

- Characteristics of the recipient, for instance whether the recipient possesses a sufficient number of the constellation of traits that it could be a potential human or mammalian pathogen.

- Existing traits in the recipient organism that might lead to an increase in pathogenicity or infectivity when altered (*e.g.* alteration of host range or tissue tropism).

- The likelihood that any disabling mutation within the recipient might be overcome, for example through complementation or reversion, due to the insertion of the foreign DNA, or through the inserted gene encoding an enzyme that would complete an anabolic pathway for a pathogenicity determinant.

- The transmissibility of the vector used to introduce relevant genes.

- Whether the foreign DNA carries a pathogenicity determinant from a related organism (toxin, invasin, integrin, and surface structures such as fimbriae, LPS and capsule).

- When the foreign DNA does carry a virulence factor, the feasibility that this gene could contribute to the pathogenicity of the genetically engineered micro-organism, or whether the virulence factor provides resistance to host defense mechanisms.

- Whether the foreign DNA carries a gene that renders the recipient resistant to an antibiotic, especially if the specific resistance has not yet spread by natural processes to the genus to which the recipient belongs (see Appelbaum, 2006, Noble *et al.*, 1992), or if the mechanism of resistance has emerged newly, like Qnr determinants (Nordmann and Poirel, 2005).

- Whether susceptibility to antibiotics or other forms of therapy may be affected as a consequence of the genetic engineering.

- Whether attenuated or inactivated strains remain stably attenuated or inactivated.

- Whether a surface component that might bind to a different receptor than that used by recipient micro-organism could increase virulence.

- Whether the foreign DNA encodes gene products, *e.g.* toxins, that even in the absence of live organisms, may cause pathogenic effects.

- Whether the derived from unrelated bacteria. foreign DNA encodes a protein that does not interact with the pathogenic properties of the parental strain but may cause pathogenic reaction, *e.g.* a modulator of growth (hormone, cytokine), or other protein with a potentially harmful biological activity (see also Bergmans *et al.*, 2008).

- When mutations are introduced that inactivate specific virulence factors of potential pathogens, whether the stability of the mutation has been demonstrated, and whether attenuation has been shown to be sufficient so that the resulting strain can be considered non-pathogenic.

112.   The above points are illustrative rather than inclusive. Assessors must use good judgment in utilising this list, recognising that additional examples may be pertinent to the case at hand. Although there are a number of considerations that must be made in the evaluation of the pathogenic potential of genetically engineered bacteria, it is highly unlikely that a pathogen will be inadvertently created from a nonpathogen lacking most or the entire constellation of traits enabling the pathogenic lifestyle by combining virulence factors.

SECTION V - REFERENCES

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