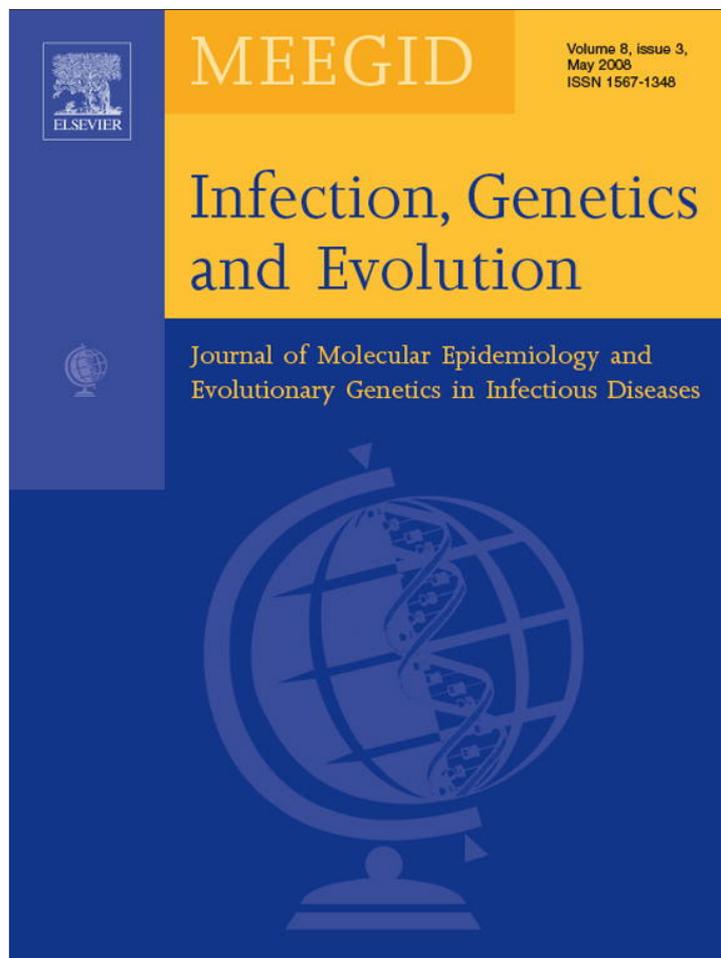


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## Discussion

## Pathogenomics: An updated European Research Agenda

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## Abstract

The emerging genomic technologies and bioinformatics provide novel opportunities for studying life-threatening human pathogens and to develop new applications for the improvement of human and animal health and the prevention, treatment, and diagnosis of infections. Based on the ecology and population biology of pathogens and related organisms and their connection to epidemiology, more accurate typing technologies and

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be used for the development of new targets for anti-infectives and therapeutic treatment. The further development and improvement of sequencing and metagenomic as well as metabolomic technologies is a prerequisite for future research, applications and novel diagnostic approaches in the field of pathogenomics.

Genome studies allowed the discovery of a wealth of unknown genes that may become targets for interfering with metabolism and signalling pathways. The improved understanding of the importance of metabolic traits for the viability and colonisation ability of bacterial pathogens within their hosts will lead to the identification of suitable metabolic targets and pathways. In particular, pathways that may be specific for groups of bacteria or single species would be promising metabolic targets to explore their interference with growth or survival of bacteria within the host and to develop novel drug targets.

Furthermore, the identification of unculturable microorganisms from the commensal flora of the host and the environmental reservoir with culture-independent methods, such as PCR amplification from microbial community DNA (metagenome) and functional or sequence-based screening of metagenomic DNA libraries will contribute to the description of complex bacterial communities, the dissection of environmental ecosystems and the biotechnological exploitation of this vast gene pool.

To facilitate the identification of antigenic diversity and variation, genomes of pathogens will be compared with those of non-pathogenic related strains. Genomes of isolates to be studied will be compared with reference genomes (i.e. completely sequenced genomes). Genomic variability and antigenic diversity of pathogenic strains will also be addressed by analysing single nucleotide polymorphisms (SNPs) and by microarrays representing whole-genomes of diverse species or strains. Comparative genomics will lead to a better understanding of the mechanisms underlying bacterial variability responsible for ongoing evolution of bacterial antigenic diversity. Prominent examples already applied successfully to comparative genomics are the studies of different *Listeria* (Hain et al., 2007) and *Escherichia coli* (Brzuszkiewicz et al., 2006) species. The entire genome of corresponding model organisms has been sequenced and compared with the genome sequence of different representative strains of the genus. In the genus *Listeria*, genome reduction has led to the generation of non-pathogenic species from pathogenic progenitor strains. It has been shown that genomic differences between uropathogenic *E. coli* are mainly restricted to large pathogenicity islands.

The knowledge of antigenic variations will allow the development of vaccines to tackle the pathogenetic mechanism of molecular mimicry of infectious bacteria associated with autoimmune disease.

Many pathogens become increasingly resistant to available drugs and antibiotics. The prevalence of antibiotic resistances is increasing in developed as well as in developing countries. They impose an important socio-economic burden to the public, industry and the health care system. Therefore, comparative genomics will be used for a better understanding of genome plasticity, gene pools, the transfer of virulence and resistance

determinants as well as the development of new treatment and prevention strategies to reduce hospital infections.

## 2.2. Evolution of microbial pathogens and antibiotic resistances

Horizontal gene transfer is a topic of major health concern and is implicated in the spread of virulence-associated and antibiotic resistance genes among bacteria contributing to the evolution of bacterial pathogens (Wright, 2007). Using Gram-negative (e.g. extended-spectrum- $\beta$ -lactamase-resistant *E. coli*) and Gram-positive (e.g. Vancomycin-resistant Enterococci, Methicillin-resistant Staphylococci) model systems, different aspects of the evolution of microbial virulence and spread of antibiotic resistances will be studied by comparative genomics and functional studies.

The mechanisms conferring the development and spread of antibiotic resistances among bacteria as well as the bacterial gene expression in response to the exposure to antibiotics will be investigated in order to get a deeper insight into the effect of antibiotics on gene regulation. These approaches will result in an improved understanding of the molecular mechanisms contributing to the development and spread of antibiotic resistances and to the discovery of novel anti-infectious agents and their targets.

Research at the molecular level on the influence of lifestyle and environmental conditions on genome plasticity and variability of bacterial pathogens will elucidate the mechanisms involved in transfer and mobilisation of antibiotic resistance genes between different bacterial strains. The improved understanding of triggering stimuli and the molecular mechanisms underlying genomic variability and the evolution of microbial virulence and antibiotic resistances will allow the development of comprehensive diagnostic tools which will enable an effective therapy adapted to the individual patients' situation (Fig. 2).

## 2.3. Microbe–microbe interactions

Microbial communities such as biofilms are involved in many infections in humans, often resulting in chronic states that are very difficult to combat (Reisner et al., 2005). Control of biofilm formation (Fig. 3) is therefore a major concern with both important health and economic issues. One of the main aims is to characterize biofilm aspects of important infections, e.g. *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, by studying the general physiological properties of bacteria associated with biofilm infections. Single pathogenic bacterial biofilms based on model systems for serious human infections will be developed and used to analyse their general phenotypic properties and gene expression profiles.

As it has been shown that the presence of specific genes and surface proteins is important for biofilm formation (Beloin et al., 2006; Valle et al., 2007), the characterization of the genetic basis and the expression profile of pathogenic bacterial biofilm formation as well as microbe–microbe interaction will lead to the identification of further factors expressed within biofilms. These factors will be utilized for the development of

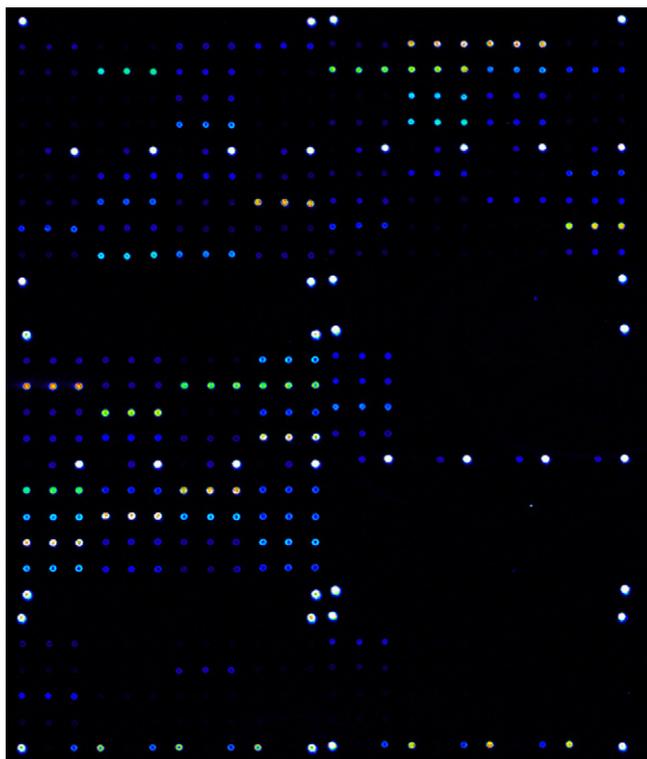


Fig. 2. Genotyping DNA chip for the simultaneous assessment of antibiotic resistance and pathogenicity potential of extraintestinal pathogenic *Escherichia coli* (false color fluorescence image). Source: T. Barl and T.T. Bachmann.

new strategies for diagnosis, prevention and control of microbial infections.

### 3. Host–microbe interactions

The complex interaction between a microbial pathogen and a host (Fig. 4) is the underlying basis of the infectious disease.

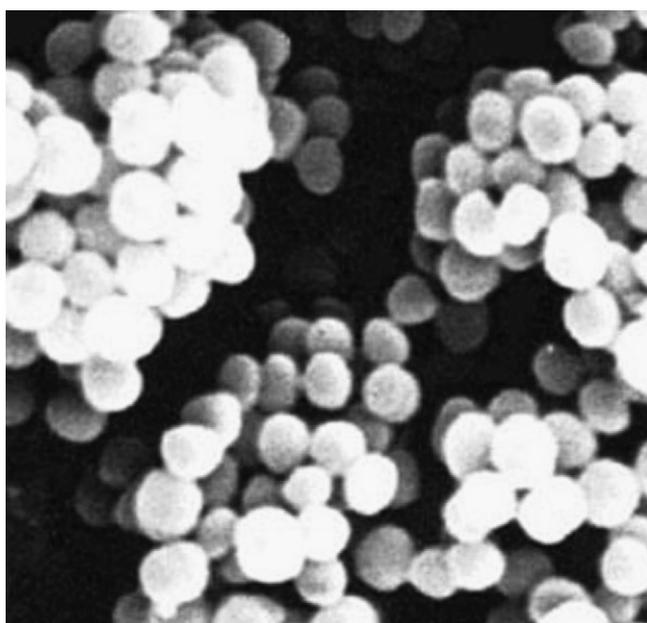


Fig. 3. *Staphylococcus epidermidis* biofilm formation. Source: H. Merkert.

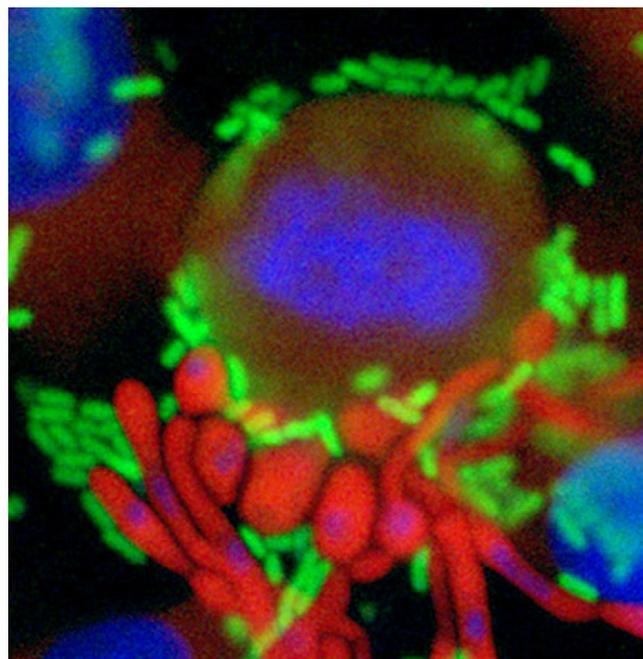


Fig. 4. FISH (fluorescence *in situ* hybridization) detection of human epithelial bladder cells (T24), *Citrobacter freundii* and *Candida albicans*. Source: H. Merkert.

The understanding of the molecular and cellular details of these host–microbe interactions may lead to the identification of virulence-associated microbial genes and host-defence strategies. This information will be used for the design of a new generation of medical tools.

#### 3.1. Metabolic interactions, gene expression and adaptation processes

Major pathogens have developed a variety of strategies with which they adapt their genetic expression to meet the challenges of their ever-changing surrounding environment, e.g. within the host cell. These include specific sigma factors, two-component systems, repressors, positive regulators, as well as small regulatory RNAs. Alone or in combination, these functions enable bacterial cells to communicate with their environment, their hosts, and with each other, allowing the bacteria to adopt specific responses, express specific proteins (toxins, adhesins, invasins, siderophores) or develop specialized structures such as biofilms or spores to ensure survival, colonisation of their ecological niches and dissemination. Studies of these metabolic interactions and adaptations in both the bacteria and the host cell will lead to a better understanding of the mutual reactions in the course of infection.

The aim is to carry out an extensive analysis of the gene activity of infected hosts and microbes during the course of infection. Studies on regulatory networks involved in the production of virulence factors and survival of pathogens *in vitro* and within the host will be accomplished by whole genome expression profiling using DNA arrays, expression studies and reporter gene fusions. Random mutagenesis or overexpression of genes will also be used in combination with

proteome analysis. Furthermore, protein–protein interaction mapping will be undertaken as well to define the structure of complex regulators and regulons. Besides a better knowledge of the molecular mechanisms essential for host responses and virulence, the discovery of new targets for antimicrobial compounds is expected.

Pathogenic microorganisms must be able to adapt to changing environmental conditions to ensure survival and growth in different host niches. This adaptation is achieved by the regulated expression of appropriate sets of genes whose products are needed by the pathogen at a particular stage of an infection in response to corresponding environmental signals. During chronic infection pathogen adaptation to specific host habitats can occur by conversion to mutator state (e.g. mismatch repair deficiency in *P. aeruginosa*) resulting in hypervariability of the genome (Hogardt et al., 2007). In addition, many pathogens have the capacity to generate variants with altered properties and to express certain products in a ‘phase-variation’ mode. Such variability enables evasion from the innate and adaptive host immune defences, e.g. *Bacillus anthracis* (Baldari et al., 2006), *Staphylococcus aureus* (van Belkum, 2006). The study of these evasion mechanisms and the development of technologies to analyse infections and corresponding gene expression on the single cell level will elucidate bacterial adaptation processes. For example, asymptomatic *E. coli* carrier strains in urinary tract infections (Fig. 5) are capable of evading the innate immune responses. These strains are adapted for growth and carry virulence genes that are not expressed (Svanborg et al., 2006).

### 3.2. Host susceptibility and interference with host cell functions

Microbial organisms may suddenly emerge as disease threats by acquiring new capacities for initiating infections and disease or by altering the host’s natural ability to mount an effective immune response. From the perspective of the host, non-specific factors as well as inherent factors related to host susceptibility or resistance, provide strategies of resistance to the changing patterns of microbial infectivity and virulence.



Fig. 5. S-Fimbriae expressing *E. coli* strain. Source: H. Merkert.

Thus, infection and immunity are always in a dynamic balance determined by characteristics of both the pathogen and the host. The study of the requirements to shift this balance between health and disease will lead to the knowledge of corresponding predictive markers. *Mycobacterium tuberculosis* is a prominent example of such bacteria that have the ability to modulate and manipulate the host immune system. Therefore, improved identification of predictive markers for quick, accurate and early detection of such bacteria will strengthen the efforts to combat bacterial pathogens (Chakhaiyar et al., 2004; Banerjee et al., 2004).

Research on infections caused by pathogenic microorganisms (bacteria and fungi) with the capacity of affecting human health is to be extended using the *in vivo* natural setting. In order to prevent infections, methods to interfere with host cell functions, e.g. by usage of RNA interference (RNAi) in the course of acute and chronic infections will be applied. Examples include acute bacterial sepsis and meningitis, characterized by an overwhelming host response, and chronic chlamydial infections with a potential of secondary pathologies. In the future, a global screening and analysis of host cell functions will enable the assessment of identified host cell functions in the living animal using *in vivo* RNAi and the development of treatment regimens. However, any *in vivo* studies and potential treatment options require prior knowledge of host cell factors, displaying essential functions in the infection process. RNAi-based loss-of-function strategies promise to give such insights, particularly with respect to the primary host cell targets of infectious agents as well as the crucial host defence machinery.

### 3.3. Cellular microbiology

The epithelial and endothelial surfaces of hosts form a physical barrier that is impermeable to most infectious agents. However, some bacteria have developed mechanisms to break these barriers (e.g. connective tissue, blood–brain barrier, gut epithelium, pulmonary epithelium, placenta) provoking severe infections. Elucidation of these mechanisms will lead to further knowledge on how to prevent the breakage of the first line of defence against microorganisms. These studies include the following topics: receptors and cell surface structures of the host cell; cell and tissue tropism; bacterial cell surface structures; cell–cell communication; specific target ligand design.

### 3.4. Commensal flora, mixed and nosocomial infections

Both humans and animals have a multitude of microenvironments such as the intestine, skin and dental cavities that are populated by phylogenetically complex microbial communities. To assess the role of the commensal flora as a protective barrier against invading pathogens but also as a reservoir for the recruitment of new emerging infectious diseases, it is important to elucidate how commensal organisms or probiotics can be used to prevent or treat infections. The evaluation of host response patterns upon exposure to a single pathogen vs. commensal flora will contribute to the understanding of the interactions of the resident flora with the host, the relation

between pathogenic and non-pathogenic species as well as the interactions among pathogens themselves. We can predict that in near future, it will be shown that many gastrointestinal disorders have bacterial components of etiology. Further efforts are required to analyse the communication between the intestinal cells and the resident flora.

The topic of secondary pathologies such as chronic infections, cancer and autoimmune diseases that may also be triggered by microorganisms as well as persistent asymptomatic infections established by selected bacterial pathogens, such as *Helicobacter pylori* and *M. tuberculosis*, in mammalian hosts will also be under scrutiny in the future.

Although not a general phenomenon, some pathogens exacerbate the effects of others. Outstanding examples are the potentiation of bacterial infections by existing viral infections. Co-infections involving various combinations of pathogens are frequently described, and some tend to be particularly severe. Diseases caused by mixed Papilloma virus–Chlamydia (Finan et al., 2006), HIV–Mycobacteria (Djoba Siawaya et al., 2007) and Influenza–*Staphylococcus* infections are major examples of these so-called mixed infections that will also be in the focus of the research agenda.

Natural and acquired factors are leading to resistance to antimicrobials of nosocomial bacteria. Production of inactivating enzymes is the most common mechanism in Gram-negative bacteria. In Gram-positive bacteria, the main mechanism involves modifications in the bacterial targets of antimicrobials. The presence of other mechanisms is common, and as a result many nosocomial strains are multiresistant. Therefore, the study of underlying mechanisms is required to overcome bacterial resistance strategies involving novel diagnostic and therapeutic approaches.

#### 4. Development and improvement of tools for research and application

In order to identify novel targets for the eradication of and vaccination against pathogens new tools, methods and bioassays as well as novel diagnostic approaches and new *in vitro* screening techniques (e.g. small bioactive molecules) have to be developed and improved for research and application.

##### 4.1. New imaging techniques

To gain further insights into the dynamic processes occurring *in vivo* and to be able to visualize and follow infections within the host, bioluminescence and other imaging techniques are required. Suitable imaging techniques will be established and used to study infectious processes in living cells, tissues and hosts and to address questions concerning cell biology, cell/tissue organisation and localisation of pathogens *in vivo*. Bacterial strains to be scrutinized will be tagged (construction of reporter strains) with appropriate markers in order to enable their three-dimensional visualisation within the living cells, tissues and hosts. The pathogen–host interaction, e.g. dynamics of the host cell cytoskeleton and membrane trafficking upon invasion of intracellular bacteria, will then be followed by different dynamic microscopic and imaging techniques including confocal microscopy and bioluminescence digital imaging. Furthermore, these techniques will allow the bioluminescent measurement of biological activities (e.g. promoter activities) inside cells and/or tissues and the localisation of virulence factors, toxins and whole bacteria in host tissues. For example, the localisation of *S. aureus* during the infection of soft tissue in the mouse has already been demonstrated successfully (Fig. 6).

The usage of structural biology as a tool to study host–microbe interactions on the molecular basis will enable a detailed understanding of microbial effectors and of host cell targets at the molecular level. This strategy has already been used to reveal the structural basis for host tropism of *Listeria monocytogenes* (Schubert et al., 2002). The establishment of relevant infection models and *in vivo* imaging will allow the detection and analysis of targeted cells.

##### 4.2. Transcriptome and proteome analysis

In order to gain new insights into host responses of infected tissues, a new generation of microarrays (amongst others custom made commercial chips, protein arrays, bacterial clone arrays, cell arrays, lab on chip) and proteomics will be used to deal with the difficulties to integrate data from multiple experiments. Already accomplished analysis of regulatory networks involved in the production of virulence factors and survival of pathogens,

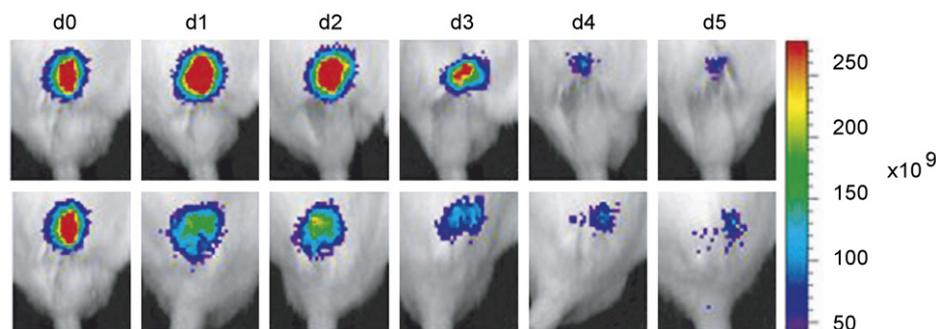


Fig. 6. Bioluminescence of *Staphylococcus aureus* in the subdermal infection model. The time course of bioluminescence was monitored for 5 consecutive days (d0–d5) after subdermal infection of the lower back area of mice with  $1 \times 10^6$  CFU *S. aureus* Xen29 (upper row) or  $1 \times 10^6$  CFU *S. aureus* *isaA* deletion mutant (second row). Signal intensity is indicated by a pseudocolor scale. Source: K. Ohlsen.

e.g. *Legionella* (Brüggemann et al., 2006) and *Pseudomonas* (Ventre et al., 2006) will be continued. These innovations with DNA array analysis in pathogenomics and infectious disease will allow the development of excellent research tools for comparative genome analysis, typing of bacterial strains and diagnostics as well as the development of biomarkers.

The transcriptome analysis of the microbe (regulating networks *in vitro* and *in vivo*) and the host (cellular and animal studies, human samples, e.g. blood) will allow the identification of novel virulence factors and new pathways specific for eukaryotic cell types, respectively. Furthermore, additional results will be achieved by combining bacterial and host transcription profiles and the integration of regulatory pathways and the cell cycle by the analysis of mutant strains.

#### 4.3. Development of delivery systems and transgene techniques

The development of recombinant vaccines by the expression of foreign antigens in attenuated strains derived from bacterial pathogens and in non-pathogenic commensal bacteria aims to stimulate mucosal immunity (Schoen et al., 2004). Mucosal immunity represents the range of host defences that prevent the attachment and invasion of infectious disease agents at the body surface of the respiratory, digestive and reproductive tracts. As the application of technologies for the generation of human vaccines is still limited, the development of new and improved bacterial delivery systems is required.

The characterisation of host response to bacterial infection and the improved understanding of the role of eukaryotic (host) factors for infection require gene knock-out techniques as well as transgenic techniques. The effects of over-expression, inappropriate expression or lack of expression of specific eukaryotic genes will be studied in order to comprehend how their encoded products confer susceptibility and also in the infection process. Therefore, animal models by transgene techniques (e.g. lacking or expressing receptors of interest) will be used to study the importance of these receptors for infection of bacterial pathogens.

#### 4.4. Strain collection and databases

The establishment of strain and tissue collections will facilitate and accelerate the exchange of biological material between researchers in the field of pathogenomics. In a future perspective, the collection of data and storage in central databases will allow rapid search and secure access to suitable biological material.

The establishment of integrated databases (genome sequences, transcriptomes, proteomes) and data analysis techniques will lead to metabolic network reconstructions (network modelling) to understand how a microorganism functions inside of the host cell. The usage of predictions generated from metabolic reconstruction models will be applied for the development of novel drug delivery methods.

In summary, the following technologies and infrastructure research will develop in the coming years which will play a

decisive role in global efforts to combat infectious diseases both emerging and re-emerging: new bioassays; imaging techniques (bioluminescence); analysis of infections and gene expression on the single cell level; RNAi technologies; novel diagnostic approaches; sequencing and metagenomics technologies; *in vitro* screening techniques; bacterial delivery systems; structural biology on the molecular basis; microarrays, proteomics of infected tissues; transgene techniques; strain and tissue collection; data analysis techniques.

## 5. Outlook

The study of microbial pathogenesis, parasitism, symbiosis, and commensalism is a large field of research requiring extensive expertise in many different domains, including genomics, epidemiology, immunology, cellular biology, and imaging techniques. To cover this field it is necessary to federate European capacities of important research centers, laboratories and industry. Therefore, the EU-funded projects Network of Excellence “EuroPathoGenomics” (NoE EPG) and ERA-NET PathoGenoMics evolved the European Research Agenda to establish an area of excellence in research on infectious diseases caused by bacterial pathogens.

The implementation of the European Research Agenda will promote discoveries leading to: (a) the development of innovative diagnostic tools, (b) the discovery of novel anti-infectious agents and their targets, (c) the identification of new antigens, and (d) the deciphering of host defence mechanisms. The detection of suitable new targets for vaccination and therapy and the development of new vaccine candidates, therapeutic strategies and diagnostic tools for the identification of virulence factors, drug targets or vaccine candidates are the long-term goals expected from the joint efforts in the future.

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