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Title:

Pathogenomics: An Updated European Research Agenda

Andreas Demuth[1], Yair Aharonowitz[2], Till T. Bachmann[3], Gabriele Blum-Oehler[1], Carmen Buchrieser[4], Antonello Covacci[5], Ulrich Dobrindt[1], Levente Emödy[6], Arie van der Ende[7], Jonathan Ewbank[8], Luis Ángel Fernández[9], Matthias Frosch[10], Francisco García-del Portillo[9], Michael S. Gilmore[11], Philippe Glaser[4], Werner Goebel[12], Seyed E. Hasnain[13], Jürgen Heesemann[14], Khalid Islam[15], Timo Korhonen[16], Martin Maiden[17], Thomas F. Meyer[18], Cesare Montecucco[19], Eric Oswald[20], Julian Parkhill[21], M. Graciela Pucciarelli[9], Eliora Ron[2], Catharina Svanborg[22], Bernt Eric Uhlin[23], Sun Nyunt Wai[23], Jürgen Wehland[24], Jörg Hacker[1*]

[1] Institut für Molekulare Infektionsbiologie, Röntgenring 11, 97070 Würzburg, GERMANY
[2] Department of Molecular Microbiology and Biotechnology, Tel Aviv University The George S. Wise Faculty of Life Science, Ramat Aviv, 69978 Tel Aviv, ISRAEL
[3] Division of Pathway Medicine, University of Edinburgh, Edinburgh, UNITED KINGDOM
[4] Unité GMP, Institut Pasteur, Département des Génomes et Génétiques, 28 rue du Dr Roux, 75724 Paris Cedex 15, France
[5] Novartis Vaccines, Via Fiorentina 1, 53100 Siena, ITALY
[6] Department of Medical Microbiology and Immunology, Szigeti ut 12, 7624 Pécs, HUNGARY
[7] Academic Medical Center, Department of Medical Microbiology, Reference Laboratory for Bacterial Meningitis, PO Box 22660, 1100 DD Amsterdam, THE NETHERLANDS
[8] Centre d'Immunologie de Marseille-Luminy, Université de la Méditerranée, Parc Scientifique de Luminy, Case 906, 13288 Marseille Cedex 9, FRANCE
[9] Centro Nacional de Biotecnología – CSIC, Campus de Cantoblanco, Darwin, 3 Madrid 28049, SPAIN
[10] Institut für Hygiene und Mikrobiologie, Josef-Schneider-Straße 2 / Bau E1, 97080 Würzburg, GERMANY
[11] Department of Ophthalmology, Harvard Medical School and The Schepens Eye Research Institute, 20 Staniford St., Boston, MA 02114, USA
[12] Lehrstuhl für Mikrobiologie, Biozentrum der Universität Würzburg, Am Hubland, 97074 Würzburg, GERMANY
[13] University of Hyderabad, Central University P.O. Hyderabad - 500 046, INDIA
[14] Max von Pettenkofer-Institut, Pettenkoferstraße 9a, D-80336 München, GERMANY
[15] Arpida Ltd, Headquarters, Research and Development, Duggingerstrasse 23, 4153 Reinach, SWITZERLAND
[16] General Microbiology, Department of Biological and Environmental Sciences, Faculty of Biosciences, P.O.Box 56, 00014 University of Helsinki, FINLAND
[17] The Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford, OX1 3SY, UNITED KINGDOM
[18] Max Planck Institute for Infection Biology, Dept. of Molecular Biology, Charitéplatz 1, 10117 Berlin, GERMANY
[19] Department of Biomedical Science, University of Padova, Viale G. Colombo 335127 Padova, ITALY
[20] INRA UMR1225, Ecole Nationale Veterinaire de Toulouse, 23 chemin des Capelles BP 87614, 31076 Toulouse Cedex 3, France
[21] The Sanger Institute, Wellcome Trust Genome Campus, Hinxton, CB10 1SA, UNITED KINGDOM
[22] Section of MIG, Sölvegatan 23, SE-223 62 Lund, SWEDEN
[23] Department of Molecular Biology, Umeå University, 90187 Umeå, SWEDEN
[24] Helmholtz-Zentrum für Infektionsforschung, Abteilung Zellbiologie, Mascheroder Weg 1, 38124 Braunschweig, GERMANY

[*] Corresponding author, Tel. 0049-931-312575, E-mail: j.hacker@mail.uni-wuerzburg.de

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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 approaches will lead to better means of disease control. The analysis of the genome plasticity and gene pools of pathogenic bacteria including antigenic diversity and antigenic variation results in more effective vaccines and vaccine implementation programs. The study of newly identified and uncultivated microorganisms enables the identification of new threats. The scrutiny of the metabolism of the pathogen in the host allows the identification of new targets for anti-infectives and therapeutic approaches. The development of modulators of host responses and mediators of host damage will be facilitated by the research on interactions of microbes and hosts, including mechanisms of host damage, acute and chronic relationships as well as commensalisms. The study of multiple pathogenic and non-pathogenic microbes interacting in the host will improve the management of multiple infections and will allow probiotic and prebiotic interventions. Needless to iterate, the application of the results of improved prevention and treatment of infections into clinical tests will have a positive impact on the management of human and animal disease.

The Pathogenomics Research Agenda draws on discussions with experts of the Network of Excellence “EuroPathoGenomics” at the management board meeting of the project held during 18 – 21 April 2007, in the Villa Vigoni, Menaggio, Italy. Based on a proposed European Research Agenda in the field of pathogenomics by the ERA-NET PathoGenoMics the meeting’s participants updated the established list of topics as the research agenda for the future.

1. Introduction

Bacterial infections remain a major cause of disease and mortality in humans and animals throughout the world. Only the detailed understanding of their pathogenic processes will provide the innovative tools for their treatment, prevention and eradication. New concepts laid down in this Research Agenda contribute to a global policy of control of infections both in Europe and in the developing world. Several infections constitute novel and particularly onerous threats owing to the occurrence of new virulent strains and the development of antibiotic resistances. Innovations in diagnostic techniques and therapy, as well as the development of vaccines against pathogenic microorganisms, are expected to come out of the joint research activities recommended in the European Research Agenda in the field of pathogenomics.

Global approaches require technical platforms (i.e. genomics, microarrays, proteomics, imaging, structure, novel bioassays) that exceed the capacities of individual laboratories or institutions including the adaptation of international standards (i.e. MIAME (Gene expression), MIAPE (proteomics), MIARE (RNAi)). To that end, this proposed agenda will join together established European groups of the Network of Excellence "EuroPathoGenomics" as well as the ERA-NET PathoGenoMics to foster the development of new multidisciplinary paradigms in the study of infectious diseases.

2. The Microbes

In order to enable the development of novel diagnostic tools, therapeutic agents and vaccine candidates it is necessary to characterize the molecular and cellular basis of infection caused by bacterial pathogens. Therefore, the following methods, techniques and research topics on microorganisms constitute the focus of the agenda:

2.*1 Genomic Tools and Comparative Genomics*

The application of genomic tools (e.g. metagenomics, sequence based typing) allows the examination of microbial ecology and population biology of pathogens and related organisms. Sequence based typing enables the analysis of genetic variation within microbial species (Brehony et al., 2007; Gutierrez et al., 2006). The understanding of horizontal gene transfer and recombination as common mechanisms in bacterial populations leading to high variability in genome size and gene content will be utilized as better means of disease control as well as for the identification and prediction of potential emerging pathogens. The use of metagenomics (Fig. 1) and metabolomics to analyse the diversity and potential activity of microbial populations will contribute to our understanding of highly prevalent, but previously unknown, metabolic processes in natural microbial communities that can 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 *E.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-β-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 analyze 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 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. 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 *Pseudomonas 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. 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 micro-organisms (bacteria, 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-off-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.

2.3 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 a near future, it will be

shown that many gastrointestinal disorders have bacterial components of etiology. Further efforts are required to analyze 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 *Mycobacterium 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 *Staphylococcus 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, 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 surfaces 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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Legend:

Fig.1: Comparison of different *E. coli* genomes. Circles represent complete *E. coli* genomes. From inside to outside: *E. coli* K-12 strain MG1655, uropathogenic *E. coli* strain 536, uropathogenic *E. coli* strain CFT073, enterohemorrhagic *E. coli* strain EDL 933. The conserved *E. coli* gene pool is indicated in grey. Pathotype-specific genes are marked in red (UPEC) or green (EHEC).
Source: E. Brzuszkiewicz, H. Brüggemann & U. Dobrindt

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

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

Fig. 4: FISH (Fluoresence *In Situ* Hybridization) detection of human epithelial bladder cells (T24), *Citrobacter freundii* and *Candida albicans*.
Source: H. Merkert

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

Fig. 6: Bioluminescence of *Staphylococcus aureus* in the subdermal infection model. The time course of bioluminescence was monitored for 5 consecutive days (d0 to d5) after subdermal infection of the lower back area of mice with $1x10^6$ CFU *S. aureus* Xen29 (upper row) or $1x10^6$ CFU *S. aureus isaA* deletion mutant (second row). Signal intensity is indicated by a pseudocolor scale.
Source: K. Ohlsen

Figure 1:


leuX
aspV
pheU
PAI II (leuX)
PAI III (thrW)
selC (LEE)
selC
PAI I (selC)
thrW
argU
serW
serX
PAI V (pheV)
pheV
argW
PAI IV (asnT)
asnT
asnW

Figure 2

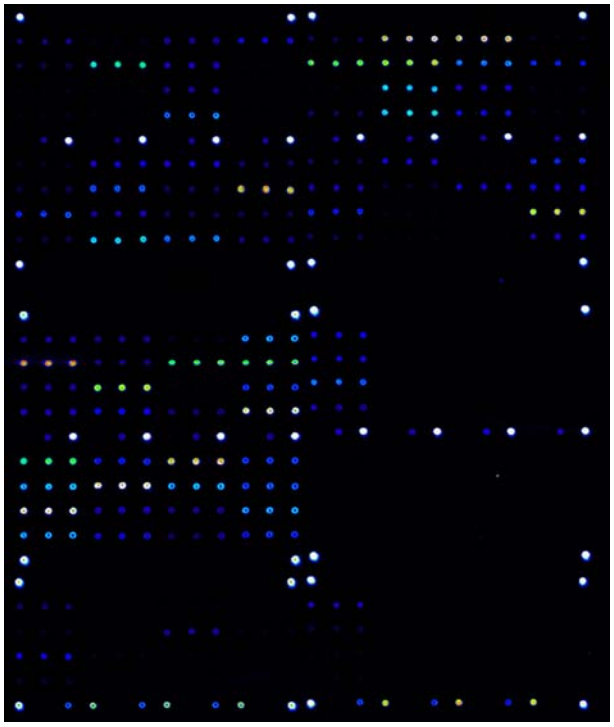

Figure 3

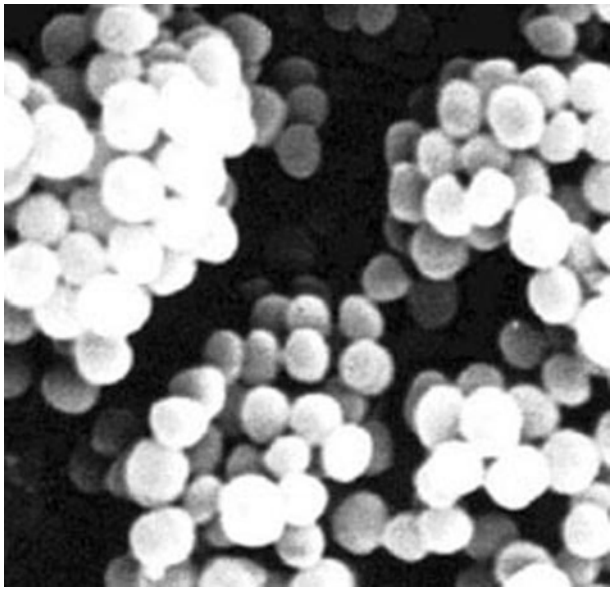

Figure 4

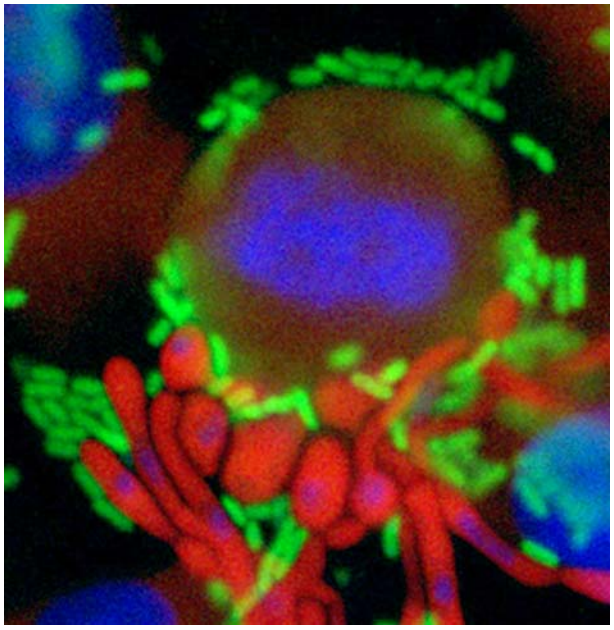

Figure 5

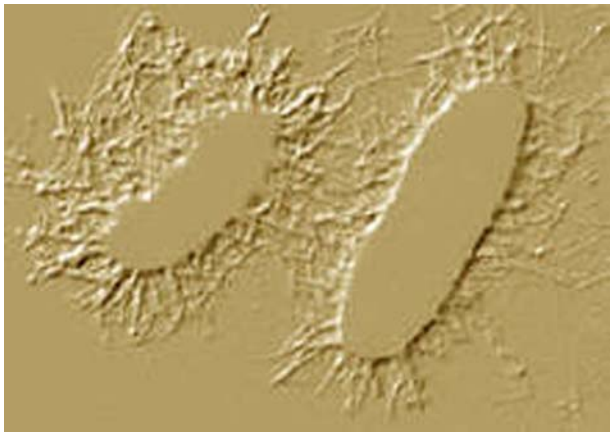

Figure 6