Functional genomics

of food-borne pathogens

Habilitationsschrift

vorgelegt von

Thilo M. Fuchs

Lehrstuhl für Mikrobielle Ökologie

Department für Biowissenschaftliche Grundlagen

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung,

Landnutzung und Umwelt

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Summary

The growing number of complete microbial genome sequences and the ready availability of their annotation provide a powerful data base for studying the biology of microorganisms. In this work, two distinct high-throughput approaches are described to exploit genomics of pathogenic bacteria, insertional-duplication mutagenesis (IDM) and expression profiling using the luciferase reporter system. Their genome-wide application to the food-borne pathogens *Salmonella typhimurium*, *Listeria monocytogenes* and *Yersinia enterocolitica* led to new insights into the complex world of microbial life in terms of (I) the minimal gene set, (II) intracellularly required factors, and (III) the association with invertebrates.

I) **Minimalism**: The essential gene set of *S. typhimurium* was defined by a novel genetic strategy. Small, randomly generated chromosomal fragments of this pathogen were cloned into a temperature-sensitive vector, and the resulting mutagenic library was grown under permissive conditions. Upon switching to non-permissive temperature, genes with essential functions under laboratory conditions could be trapped following discrimination between lethal and non-lethal recombination events. Further characterization of a total of ~500 fragments revealed 145 known essential genes and 112 functionally characterised or hypothetical genes not yet demonstrated to be essential for a bacterial cell; this number corresponds to approximately 11% of the *Salmonella* genome.

II) **Specialization**: More than 1,000 IDM mutants of the facultative intracellular pathogen *L. monocytogenes* were screened for their phenotypes in human epithelial cells. The genetic analysis of severely attenuated mutants revealed a huge number of listerial genes required for replication in non-phagocytic cells, thus dissecting the genome of *Listeria* in terms of their adaptation to the intracellular environment. The acquisition of species-specific genes and the usage of alternative sugar and nitrogen sources could be demonstrated as novel strategies that enable this pathogen to survive in the cytosol of the host cell.

III) **Association**: A promoter fusion library of *Y. enterocolitica* was constructed by the transposon-mediated chromosomal insertion of the luciferase reporter, and the transcriptional response of the genome was derived when cells were exposed to low temperatures. Sequence analysis revealed a novel pathogenicity island termed *tc*-PAI^{Ye} carrying insecticidal toxin genes that could be demonstrated to be transcriptionally silent at body temperature, but to be essential for *Y. enterocolitica* toxicity against insects at low temperature. This data demonstrates a yet unknown pathogenicity phase of *Y. enterocolitica* in insects, suggesting invertebrates as a potential source of pathogen evolution.

2. Introduction: functional genomics

(Appendix VIII.-X.)

Entering the genomic era

The year 1995 marks the beginning of the genomic era. It was the sequencing of the *Haemophilus influenzae* chromosome that provided access to the first entire genetic content of a microorganism (Fleischmann *et al.*, 1995). Up to date, more than 300 genome sequences of eubacteria have been made available to researchers (NCBI, homepage 2006). Despite strong efforts currently made to translate these DNA sequences into biological functions, between 36% (*Yersinia pestis*) and 65% (*Pseudomonas aeruginosa*) of all open reading frames listed for various bacterial pathogens still correspond to genes of either hypothetical or unknown function (Table 1) (Merrell and Camilli, 2002). Even in *Salmonella enterica* sv. Typhimurium (*S. typhimurium*), one of the prokaryotes studied most extensively, approximately 46% of all annotated open reading frames (ORFs) escape even tentative identification (Washington University School of Medicine St. Louis, homepage 2006). Many of those functional unknown genes are likely to be involved in bacterial survival in different natural environments, such as soil, food, and healthy or ill hosts (Schaechter, 2001). Thus, a key challenge for future microbial research is the attribution of yet uncharacterized genes to a cellular function. The sequence of a functional unknown gene and the encoded protein may suggest a possible biochemical activity due to the presence of homologies to genes, motifs or domains with known activities. Indeed, many examples from the literature show how data base searches can support the design of appropriate conventional genetic and biochemical experiments to disclose yet uncharacterized cellular functions. Unknown genes often play an important role in pathway reconstruction to complete our knowledge of bacterial metabolism. For example, the pathway of diaminopimelate (DAP) and lysine synthesis has not been completely characterized in many microorganisms. A yet uncharacterized gene of *Bordetella pertussis* was found to be transcriptionally coupled with *dapD* and *dapE* involved in the biosynthesis of DAP and lysine. Genetic and biochemical analysis revealed this gene to encode an N-succinyl-L,L-DAP aminotransferase, the missing link of the succinylase branch of lysine synthesis (Fuchs *et al.*, 2000).

The genomic era holds great promise for food safety in terms of the containment of human-pathogenic bacteria such as *Salmonella* spp., *Listeria* spp., and *Yersinia enterocolitica*. Identification of bacterial genes expressed preferentially within infected cells and animals is critical in understanding how bacterial pathogens circumvent the immune system and cause disease. Many complete genome sequences of food-borne

pathogens have been determined, mainly because due to the considerable pharmaceutical interest in novel anti-infective strategies (de Vos, 2001). In addition to assisting the development of immunization strategies or targets for diagnostic purposes, the availability of the complete genomes of food-borne pathogens also allows to analyse their response to various environmental conditions, thereby uncovering their traits of transmission.

pathogenic species	genome size [Mb]	% known function	% unknown function
Salmonella typhimurium[a]	4.86	53.5	46.5
Listeria monocytogenes[b]	2.94	64.7	35.3
Yersinia enterocolitica[c]	4.62	64.2	35.8
average[d]	-	47.4	52.6

Table 1: Percentage of unknown and functionally characterized genes in pathogens investigated in this work. [a](McClelland *et al.*, 2001); [b](Glaser *et al.*, 2001); [c]Estimation according to the *Y. pestis* genome (Merrell and Camilli, 2002); [d]The value represents the data of 87 completed genomes. The values underestimate the percentage of functional unknown genes because a function might be attributed due to sequence homologies only.

Functional genomics

"Genomics" can be defined briefly as "the combination of the complete genome sequences and the informatic tools with which to analyse them" (Strauss and Falkow, 1997). The general information derived from microbial genomics already led to a better understanding of the biology of bacteria. For example, it was found that free-living species such as *Bacillus subtilis*, *Escherichia coli* or *P. aeruginosa* commit up to 9% of their genomes to genes encoding regulators. In *Helicobacter pylori* that lives in a more specialized environment, global regulatory proteins are less abundant than in *E. coli*, among them only four sensor proteins and seven response regulators (Tomb *et al.*, 1997). However, a major limitation of genomics is that their interpretation requires information on gene function, and the information available from database searches is often misleading. Closing the gap between sequence data and cell physiology is the challenge of "functional genomics". This term describes the combination of genome-orientated studies with biological or biochemical assays, thus allowing the acquisition of experimentally qualified functional data.

Proven techniques and modern approaches used in functional genomics

Bacterial genetics has been revolutionized by the availability of whole genome sequences. While the location of an insertion mutant required laborious means like gene mapping in the pre-genomic era, the sequencing of not more than 100 bp is now sufficient to genetically address the observed phenotype (Hinton, 1997). To permit the large-scale analysis of bacterial genomes, already proven methods such as transposon mutagenesis and analysis of transcriptional activity were further improved and adapted to the needs of functional genomics (Hayes, 2003). Complementing methodologies are the analysis of protein expression patterns, metabolic profiling, comparative genomics or the application of promoter reporter fusions. Due to the medical relevance of pathogenic bacteria, the object of many of these genetic approaches is the identification of putative virulence determinants (Fuchs, 1998). Within this class of factors are those that enable the microbe to adapt to host-specific microenvironments or to evade the host's immune defence mechanisms.

IVET (in vivo expression technology) is one of the most widely applied technologies devised for identifying bacterial genes that are preferentially induced when a pathogen associates with its host cell, for example during intracellular growth in macrophages (Mahan *et al.*, 1993). This method is based on a library in which random genomic fragments are ligated to a gene that complements a defect in an auxotrophic mutant of the host strain. The pool of fusions is then transferred into the host strain and selected for integration into the chromosome by homologous recombination. Only those bacteria that harbour a promoter fusion active under conditions prevailing *in vivo* are able to survive the passage through an infected animal. However, a disadvantage of the IVET strategy is that it fails to detect genes only transiently expressed. A recent modification called RIVET ("recombinase-based IVET") uses the irreversible excision of a selectable marker to overcome this limitation, yielding genes that are required only at a specific time point during infection (Camilli and Mekalanos, 1995; Lee and Camilli, 2000). IVET has been applied to a broad variety of pathogenic microorganisms (Handfield and Levesque, 1999) such as *S. typhimurium*, *Staphylococcus aureus* (Lowe *et al.*, 1998) or *P. aeruginosa* (Wang *et al.*, 1996), and the subsequent knockout of many genes identified by this approach led to mutants significantly attenuated in virulence. It is interesting to note that many of those IVET genes do not encode classical virulence factors, but rather are involved in intermediate metabolic pathways and housekeeping functions (Merrell and Camilli, 2000).

On the basis of *differential fluorescence induction (DFI)*, an alternative strategy was developed to identify bacterial genes specifically expressed when a bacterium is associated with its host cell (Valdivia and Falkow, 1997). In contrast to IVET, DFI uncouples selection parameters from nutritional requirements or sensitivity to antibiotics and allows high-throughput screenings. Host cells infected with bacteria bearing random

transcriptional fusions to *gfp* were separated by a fluorescence-activated cell sorter based on stimulus-dependent synthesis of Gfp. The application of DFI to *Salmonella* led to the isolation of genes that respond to an acidic environment and those that are exclusively expressed within macrophages. This methodology was extended to *Streptococcus pneumoniae*, resulting in the identification of loci regulated by competence stimulatory peptides (Bartilson *et al.*, 2001), and to *E. coli*, revealing genes that help to overcome the blood brain barrier (Valdivia and Falkow, 1997). DFI was only recently improved by the use of two-colour flow cytometry that permits to derive quantitative data on *Salmonella* gene expression in infected hosts (Bumann, 2002).

The application of IVET or DFI screenings, however, will not yield a comprehensive portrait of *in vivo* expressed genes, a limitation that can be overcome by the use of *RNA microarray analysis*. The potential of this technique lies in its ability to assay the expression of thousands of genes in parallel and in a semiautomated and quantitative approach. Using RNA microarrays, transcriptome snapshots can be compiled that document gene expression temporally, such as during an infection, or spatially, in a certain tissue in the host's body. In the latter case, expression profiles of bacteria within infected tissues are compared with profiles from bacteria cultured under standardized *in vitro* growth conditions. The first study that achieved this goal unravelled the expression profile of *S. typhimurium* at different time points during proliferation in macrophages, and similar approaches in *Brucella*, *Listeria* and others have followed since then (Chatterjee *et al.*, 2006; Eriksson *et al.*, 2003; Gaynor *et al.*, 2005; Kohler *et al.*, 2002; Lucchini *et al.*, 2005; Rengarajan *et al.*, 2005). However, a major drawback of this kind of studies, as well as of the IVET strategy, is that genes induced within host cells do not necessarily contribute to a pathogen's ability to survive and replicate in this compartment.

Another powerful method, *signature-tagged transposon mutagenesis (STM)*, has been used to identify genes that are required for survival in a host (Hensel *et al.*, 1995). STM combines the strength of mutational analysis with the ability to follow simultaneously the fate of a large number of different mutants within a single animal. STM requires, like IVET, passage through a host, but instead of measuring promoter activity, it determines whether disrupting a particular gene severely affects bacterial survival. In STM, each member of a complex library of mutants is tagged with a unique DNA sequence. If a mutant is absent after passage of the library through an infected animal or another selective environment, the insertional mutation it harboured may be located in a gene essential for virulence. The first application of STM led to the discovery of a previously unknown 40-kb pathogenicity island termed SPI2 that encodes components of a type III secretion system. In the meanwhile, STM has been applied to more than 11 bacterial species, and the screenings identified several hundred putative virulence factors whose

significance for maintenance of the pathogen in the host, however, still remains to be elucidated in many cases (Lehoux and Levesque, 2000).

Comparative genomics is often used to identify putative virulence-associated genes (Middendorf *et al.*, 2005). Genomic variability is the genetic reason for phenotypic differences between non-pathogenic commensals and virulent biotypes, and natural selection results in strains and species adapted to particular microenvironments of the host (Schoolnik, 2002). Accordingly, comparisons of closely related genomes, for example those of pathogenic and non-pathogenic serotypes of the same species, can provide valuable information about the diversity and evolution of pathogens, because genes exclusively present in the pathogenic strain may be essential for infection, virulence or adaptation to a particular host niche (Ochman and Moran, 2001). Comparative genomics is performed most easily by *in silico* analysis of fully sequenced genomes, a rational approach given the low cost of high-throughput DNA sequencing. The comparison of five sequenced *Salmonella* serovars revealed, for example, that approximately 500-600 kb (10-12%) of chromosomal DNA are unique to each serovar. These regions are assumed to be responsible for the different virulence properties of *S. dublin*, *S. enteritidis*, *S. paratyphi*, *S. typhimurium*, and *S. typhi* (Edwards *et al.*, 2002). The availability of whole genomes also allows the construction of microarrays based on representations of all the ORFs of a sequenced strain that are then hybridized with labelled DNA from one or several unsequenced experimental strains. The resulting array data will particularly decipher genes that are present in the reference strain but absent in the experimental strain (Schoolnik, 2002).

Dual use of reporter fusion libraries

A more general methodology to dissect microbial genomes for novel functions is based on the luciferase reporter cassette that enables the highly sensitive measurement of promoter activities. For a genome-wide approach, the *luxCDABE* genes are randomly inserted into a bacterial chromosome upon transposon mutagenesis. If the promoterless reporter insertion is located downstream of a promoter and in sense orientation with respect to gene transcription, the expression of the gene or gene cluster driven by this promoter can easily be profiled by bioluminescence measurement. For analytical purposes, conditions of choice are then applied to the mutant library, and the induction rate of all reporter fusions can be measured over all bacterial growth phases to identify genes that are differentially regulated in comparison to standard conditions. To assign a cellular function to individual gene loci, strains exhibiting significantly induced or reduced transcriptional activity are then combined, and the derived pool of gene knockout mutants is subjected to biochemical or biological assays. This screening procedure illustrated in Fig. 1 can rapidly be extended to varying growth conditions using the same

transposon library, demonstrating that the dual use of luciferase reporter fusions is a powerful, yet under-used strategy in functional genome analysis of bacterial pathogens.

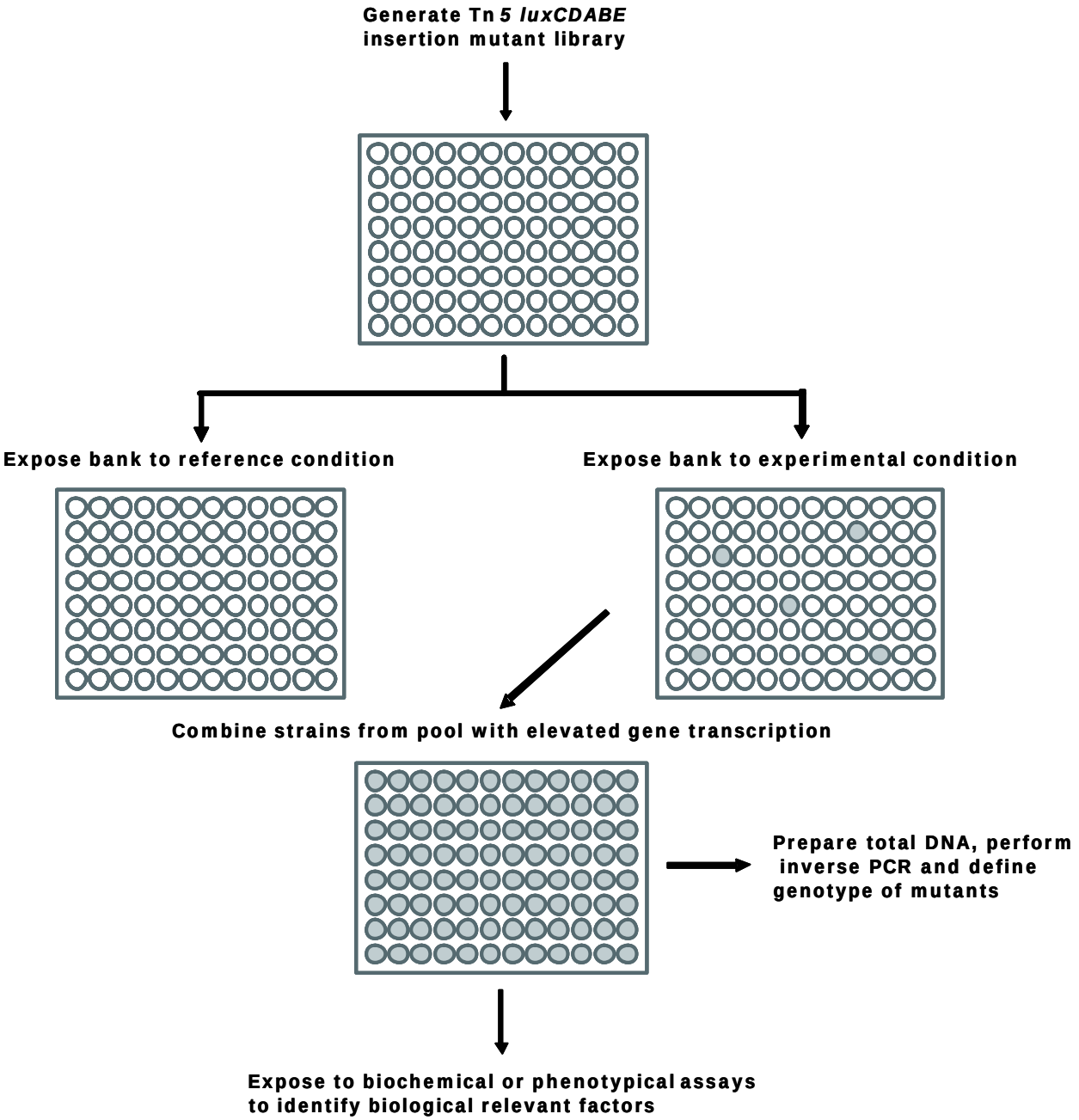

Fig. 1: Efficient strategy to assess the *in vivo* functionality of bacterial genes taking advantage of the dual character of luciferase reporter strains that can either be used for expression profiling or for phenotype characterization due to gene knockout.

3. Genome-wide insertional-duplication mutagenesis (IDM)

(Appendix VII.)

A common approach to the functional characterization of a so far uncharacterized gene is the knockout of its coding sequence and the study of phenotypic changes under varying conditions. Transposons are valuable mutagens, but often exhibit some degree of target preference and cannot be used easily to identify the insertion site.

Insertion-duplication mutagenesis (IDM)

IDM is an alternative, established method of gene knockout with the strong advantage of direct targeting of selected genes (Fig. 2). This technique requires a conditionally replicating vector that is not capable to proliferate under non-permissive growth conditions due to its temperature-dependence, or the absence of a replication protein. In general, IDM bases on homologous (Campbell-type) recombination between the bacterial chromosome and a plasmid carrying a chromosomal fragment, resulting in plasmid integration into the chromosome and fragment duplication. If the replication ability of the used plasmid is temperature-sensitive, strains that harbour the plasmid on the chromosome can be selected by growth at the non-permissive temperature, since only these cells remain resistant against the antibiotic applied.

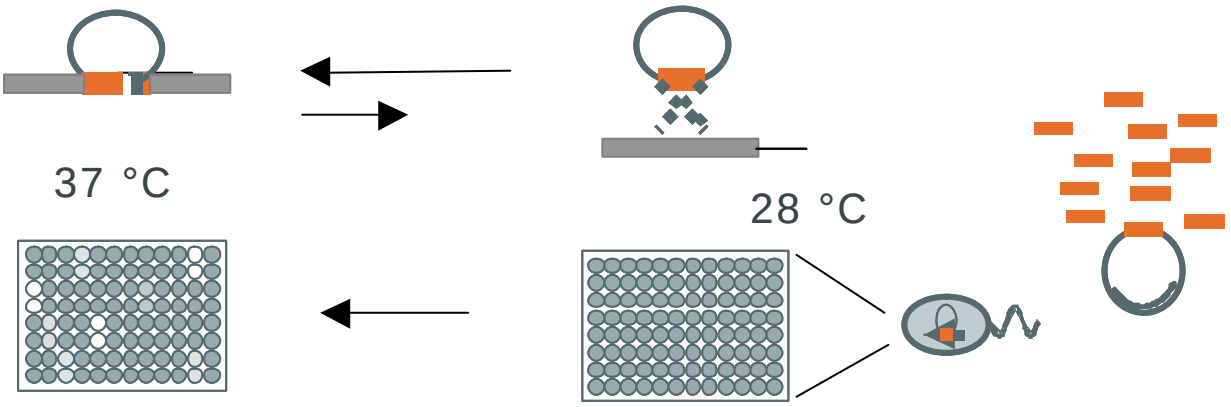

Fig. 2: Principle of homologous recombination using a temperature-sensitive vector. In some cases, no viable mutants are obtained following IDM due to gene essentiality.

Conditionally replicating vectors

Plasmids of restricted replicability are versatile tools for genetic manipulations like the delivery of transposable elements (Michiels and Cornelis, 1986), the expression of essential gene products (Dai and Lutkenhaus, 1991), the construction of plasmids for biological containment (Hashimoto-Gotoh *et al.*, 1981), or the construction of conditional mutants, which is important for studies of genes essential for cell survival (Jana *et al.*,

2000). Those vectors also offer the opportunity to site-specifically introduce promoter-probe vectors, resulting in isogenic strains without further extrachromosomal copies of the reporter gene (Vagner *et al.*, 1998).

The most prominent application of thermosensitive plasmids is gene disruption and allelic exchange with the chromosome of Gram-positive and Gram-negative bacteria. Temperature-sensitive vectors have been used to construct insertional knockouts or deletion mutants of *L. monocytogenes* (Wuenscher *et al.*, 1991), *S. pneumoniae* (Lee *et al.*, 1998), *Lactobacillus* spp. and *B. subtilis* (Biswas *et al.*, 1993; Law *et al.*, 1995). A pGK12 derivative also allowed the systematic, genome-wide construction of regulatory mutants to confirm the essentiality of *B. subtilis* genes (Kobayashi *et al.*, 2003). In all cases described, insertional knockout mutants can be obtained directly by incubating transformed strains at non-permissive temperature, or by growth at permissive conditions followed by a shift to higher temperatures. Thermosensitive mutants of pSC101-derivatives like pKO3, pMAK705, HSG422 or pHS1 are proven tools for genetic manipulations of *E. coli* and *Salmonella* spp. (Hamilton *et al.*, 1989; Hashimoto-Gotoh *et al.*, 1981; Link *et al.*, 1997; Merlin *et al.*, 2002; Phillips, 1999). Before the development of the more efficient one-step inactivation method basing on the phage λ Red recombinase (Datsenko and Wanner, 2000), those vectors were the preferred means to generate gene deletions.

Construction of a novel thermosensitive vector for insertion-duplication mutagenesis of *S. typhimurium*

Despite the proven value of temperature-sensitive derivatives of pSC101 such as pMAK700 or HSG422, they are not well suited for use in genome-wide, random gene disruption mutagenesis because pSC101 derivatives are represented by 6-10 molecules per cell only. When a mutagenic library with fragments of 200-400 bp is used, however, a high copy number rate is a prerequisite for insertional mutagenesis due to low recombination rates in the case of short homologous sequences. Therefore, the broad-host-range, thermosensitive plasmid pVE6007 derived from pGK12 was adapted for its use in Gram-negative bacteria such as *E. coli* and *S. enterica*. The resulting vector pIDM1 carries several recognition sites for restriction enzymes, encodes a tetracycline efflux mechanism, and enables blue/white selection for efficient cloning by α complementation. A low shutoff temperature of 37°C for replication was determined, as well as a minimal copy number of 228 per cell. It was further shown that integrants are highly stable at non-permissive temperature in the absence of tetracycline during *in vitro* growth, and during infection of macrophage cells (Fuchs *et al.*, 2006). pIDM1-insertion mutants can therefore be used in cell culture experiments or *in vivo* studies. It was also be demonstrated that single crossover events in *S. typhimurium* require a minimal length of 50 bp homology between the chimeric pIDM1 and its target *phoN* sequence, thus

permitting to inactivate short genes that have not yet been accessible to IDM in Gram-negative bacteria. Systematic shortening of an intragenic fragment confirmed that the recombination frequency is proportional to the third power of the homology length also in the smaller-length range.

Construction of mutagenic fragment libraries of food-borne pathogens

Despite these numerous examples that exploited IDM for the analysis of individual genes in Gram positive and Gram negative bacteria, the potential of IDM as a highly random mutagenic tool for genome-wide analyses has yet been underused. Another advantage of IDM compared to transposon mutagenesis is the identification of the target locus in a one-step procedure. In the most comprehensive approach described so far (Lee *et al.*, 1999), a large pneumococcal transformant library was derived from 20,000 mutagenic plasmids to isolate mutants defective in genetic transformation. In order to explore the genomes of *S. typhimurium* and *L. monocytogenes* for genes essential for growth in rich medium or for intracellular replication, mutagenic fragment libraries of the chromosomal DNA of both pathogens were constructed (Knuth *et al.*, 2004; Joseph *et al.*, 2006).

a) To establish a random library of *S. typhimurium*, chromosomal fragments of an average length of 350 to 450 bp were generated by a PCR method modified by Froussard (Froussard, 1992). *Kpn*I-restricted fragments were cloned into pIDM1, and the resulting library was transformed into *E. coli* EC101 cells. The redundancy of the library was determined to be lower than 10%. Pooled plasmid DNAs isolated from *E. coli* clones were then transformed into *S. typhimurium*, and transformants were cultivated at 30°C. For further use of the mutagenic fragment library, 14,000 colonies were picked individually into microtitre plates. According to the calculation of Lee *et al.* (Lee *et al.*, 1998), this library should represent approximately 62% of all genes of the *S. typhimurium* genome.

b) In a similar manner, a random fragment library of *L. monocytogenes* strain EGD was constructed using a derivative of the broad host-range plasmid pLSV1. This vector replicates in *Listeria* at 30°C, while it is lost after several cell divisions at 42°C due to two transition mutations in the *repF* gene responsible for plasmid replication. In contrast to the *Salmonella* fragment bank described above, DNA fragments of *L. monocytogenes* EGD were randomly generated by sonication according to a modified protocol of Lee *et al.* (Lee *et al.*, 1999). The fragments were then digested with *Sau*3A and ligated to *Bam*HI-restricted vector pLSV101. Ligation samples were then transformed into *E. coli* strain XL2-blue, and *E. coli* transformants were selected at 37°C on LB agar containing erythromycin. Colonies were pooled in sets of 60-380 clones, and plasmid DNA isolated from each pool was transformed into *L. monocytogenes* cells. Approximately 1,500 *L. monocytogenes* EGD clones of this mutagenic library were selected at 30°C in the presence of erythromycin and also stored in microtitre plates. Similar to the procedure

described above, insertional mutants were isolated from this mutagenic library during growth at 42°C in the presence of 5µg/ml erythromycin.

Dual use of mutagenic libraries cloned in conditionally replicating plasmids

The very nature of such a mutagenic library allows their use to i) select insertional mutants, to ii) identify lethal insertions, and iii) to restore the wild-type phenotype by plasmid excision and loss.

i) In each colony of the library harbouring a cloned fragment, homologous recombination occurred between this fragment and its chromosomal counterpart during growth at permissive temperature. The frequency of recombination events per cell varied usually between 10^{-2} and 10^{-6} depending on factors such as fragment length, vector copy number, or DNA structure and transcriptional activity of the targeted locus. To efficiently convert the *in trans* mutagenic library into a bank of insertion mutants, aliquots of microtitre cultures were dropped at the edge of an agar plate, and cells were then spread by tilting the plate to allow the drop running down the plate. Cells harbouring chromosomally integrated plasmid were selected upon growth at non-permissive temperature in the presence of the antibiotic against which the plasmid provides resistance. This high-throughput procedure resulted in two insertion mutant banks comprising approximately 9,100 strains (*S. typhimurium*) or 1,700 strains (*L. monocytogenes*).

ii) Approximately 3.6% of all *S. typhimurium* clones subjected to the IDM procedure did not yield viable insertion mutants. Since illegitimate recombination could be excluded, vector insertions probably affected genes indispensable for growth under laboratory conditions. This method was therefore applied to identify fragments that resulted in lethal phenotypes or drastically reduced numbers of viable cells in order to define the essential gene set of this pathogenic microorganism.

iii) Insertion mutants were isolated and proliferated at non-permissive temperature (37°C). When they were subsequently grown at 30°C, excision of the inserted plasmid occurred by a second recombination event that restored the wild-type genotype. The excised plasmid was either lost or recovered (Fuchs *et al.*, 2006), in the latter case allowing a rapid shift between the two phases of *in trans* replication or chromosomal integration. This procedure permits to ascribe a certain phenotype to the gene affected by IDM and not by other mutations. It could also be demonstrated that inaccurate plasmid excision probably took place in only 1.6% of all investigated clones. Excision of a thermosensitive plasmid as a result of intragenic recombination is, for two reasons, more efficient than integration by intermolecular recombination.

4. The essential gene set of *S. typhimurium*

(Appendix V., VI.)

The species *Salmonella enterica* serovar Typhimurium

The genus *Salmonella* comprises two species, *S. enterica* and *S. bongori*, with eight subspecies. Salmonellae are facultative intracellular bacteria and a major cause of diseases worldwide, among them enteric (typhoid) fever, gastroenteritis, bacteremia and localized systemic infections. *S. enterica* serovar Typhimurium (*S. typhimurium*) is one of the leading causes of food poisoning in human beings; in mice, it evokes a disseminated infection similar to human typhoid. Since *S. typhimurium* is amenable to genetic manipulation and can be evaluated in a well-characterized mouse model system, the molecular mechanisms underlying its virulence have been extensively studied (Hapfelmeier and Hardt, 2005). During the course of infection of a mammalian host, *S. typhimurium* experiences complex and changing environments including several different tissues as well as a broad range of host cell types such as epithelial cells, macrophages and dendritic cells. Studies of the pathophysiology of human and mouse typhoid fever suggest that the ability to invade epithelial cells and, even more important, to survive and replicate in the phagosome of macrophages is a hallmark of *Salmonella* pathogenesis (Fields *et al.*, 1986). The bacterial uptake into epithelial cells involves the modification of the actin cytoskeleton of the host cell and is mediated by effector proteins translocated into the eukaryotic cell via a type III secretion system (T3SS). This process enables *S. typhimurium* to efficiently penetrate the intestinal epithelial barrier and to disseminate to deeper tissues. By residing within macrophages, the pathogenic bacteria evade the host's immune response and are transported via the lymphatics and the bloodstream to the mesenteric lymph nodes and then to liver and spleen. In macrophages, *S. typhimurium* exists in a membrane-bound compartment known as the *Salmonella*-containing vacuole (SCV) (Holden, 2002). Intracellularly growing *Salmonella* express specific, well-characterized sets of genes, particular those connected with the *Salmonella* pathogenicity island 2 T3SS or with the virulence plasmid pSLT, to overcome unfavourable conditions within the host cell. The host defence mechanisms include antimicrobial activities, a low pH and a limitation for certain nutrients such as aromatic amino acids and purine bases. A prominent example for a bacterial factor activated within the vacuole is the two-component system PhoQP that confers resistance to antimicrobial peptides by modifying lipopolysaccharides (Alpuche Aranda *et al.*, 1992). In the near future, the extensive characterization of metabolic networks of *Salmonella* and closely related *E. coli* will also facilitate the systematic analysis of metabolism during infection. A major goal of the study presented here was the identification of novel antibiotic targets which are urgently desired to control and combat bacterial infectious diseases. An effective methodology

based on a trap for lethal insertions was developed and applied to *S. enterica* to identify *Salmonella* proteins encoded by genes indispensable under optimal growth conditions.

The concept of a minimal genome

A central question in current biology is: which genes is a minimal genome composed of? The concept of a minimal set of genes that are necessary and sufficient to sustain cellular life refers to the most favourable conditions imaginable, e. g. the presence of all nutrients and the absence of environmental stress (Koonin, 2000). Studying the extremely reduced genome of *Buchnera* species that live in aphid cells, it was found that only five (0.9%) genes have no homologue in *E. coli* (Gil *et al.*, 2002). Therefore, the genomes of intracellular parasites and symbionts are assumed to provide a deeper insight into the minimal genome. A comparison of the first two publicly available whole bacterial genomes, those of *H. influenzae* and *Mycoplasma genitalium*, derived a set of approximately 240 genes conserved among both organisms (Mushegian and Koonin, 1996). By adding genes to fill in the missing steps of critical metabolic pathways that are encoded by dissimilar genes in the two bacteria, and by subtracting apparently redundant and organism-specific genes, the main features of a hypothetical minimal gene set could be derived (Mushegian and Koonin, 1996). Interestingly, one third of theses genes predominately encoding translation factors, tRNA synthetases, and subunits of RNA polymerase, proved to be universal, e. g. orthologous genes are found in eubacteria, archae and eukaryotes. Another 30% revealed to be highly conserved among all bacterial species. Re-evaluation of the concept of a minimal genome led to the conclusion that it is more appropriate to consider a minimal gene set of functional niches rather than a rigid minimal genome, as it is more fruitful to investigate genes indispensable under a variety of different conditions and lifestyles such as thermophily or chemoautotrophy (Fuchs, 2005; Koonin, 2000).

Negative and positive approaches to identify essential genes

Many of the gene products required for bacterial proliferation under optimal growth conditions are likely candidates for target-based drug development. However, the minimal gene set falls short of comprising all essential genes of bacteria with larger chromosomes due to non-orthologous gene displacement, or the more complex composition of biochemical pathways and cell structures. Therefore, and because conventional antibiotics are becoming less effective, many efforts have been made to identify putative essential genes of medically relevant bacterial pathogens (Table 2). Conceptually, there are two ways to identify essential genes or regions of the bacterial chromosome: i) the negative approach, which identifies many regions that are not essential and presumes that everything else is essential, and ii) the positive approach

which identifies genes that are essential by generating a conditional mutation and demonstrating its lethal phenotype. To this end, 5´- fragments of genes of interest are cloned behind a regulable promoter such as P_{lac} within a conditionally replicating vector. Upon transformation and subsequent homologous recombination, the chromosomal copy of the putative essential gene comes under control of the artificial promoter which can be repressed or induced by an appropriate media composition (Kobayashi *et al.*, 2003). The most reliable predictions of gene essentiality base on a combination of both these approaches, and by the supplementary application of targeted gene deletion methods.

Conditional mutations and antisense RNA

Both these methods were applied particularly to identify genes essential for the growth of Gram-positive bacteria. The first approach used a collection of temperature sensitive mutants generated by chemical or UV mutagenesis (Martin *et al.*, 1999). It was supposed that gene products which can mutate to conditional lethality are essential for viability (Schmid, 1998). Mutated colonies were replica plated in duplicate and incubated either under permissive or non-permissive growth conditions. One of the major disadvantages of temperature-sensitive mutant strategies is the disability of approximately one third of proteins to achieve thermolabile mutants making it difficult to isolate conditional mutants in certain genes (Harris *et al.*, 1992; Schmid *et al.*, 1989). Furthermore, complementation of the selected mutants, sequencing of the complementing genome regions and validation of targets are time consuming.

The second approach identified critical staphylococcal phenotypes that were generated by antisense RNA (Forsyth *et al.*, 2002; Ji *et al.*, 2001). This methodology bases on a random library of small DNA fragments from *S. aureus* under control of a tetracycline transcription regulatory system. The colonies were screened upon replica plating in the presence or absence of tetracycline, thus identifying more than 650 staphylococcal genes critical for bacterial growth. A major drawback of this methodology is that the use of antisense RNA is limited to genes for which an adequate expression of the inhibitory RNA can be obtained in the organism under study.

Comparative genomics

The idea behind the application of comparative genomics to identify essential genes is that genes conserved in at least two bacteria probably encode indispensable cell functions. By comparison of the ORFs of *M. genitalium* and *H. influenzae*, two of the smallest bacterial genomes sequenced so far, it was postulated that 256 genes are close to the minimal set that is necessary and sufficient to sustain the existence of a bacterial cell (Mushegian and Koonin, 1996). A recent approach identified 27 *E. coli* ORF that are both of unknown function and conserved in the compact genome of *Mycoplasma*

genitalium. Six of the 27 ORF were then shown to be essential for *E. coli* viability (Arigoni *et al.*, 1998). Genes which are conserved in *H. pylori* spp. but highly divergent in other eubacteria, were screened using a vector-free allelic replacement mutagenesis technique, resulting in the identification of 33 essential genes (Chalker AF *et al.*, 2001). However, comparative genomics will not cover the whole set of essential nucleic acid sequences of different pathogens. Moreover, only few species-specific genes will be identified by this approach.

Systematic knockout approaches

Functional analyses of the whole genomes of the model organisms *Saccharomyces cerevisiae* or *B. subtilis* were performed by systematic investigation of every gene in one organism (Kobayashi *et al.*, 2003; Winzeler *et al.*, 1999a). Those targeted knockout approaches are very resource intensive and cannot be applied to a broad range of pathogenic microbes. However, reliable and independent data of a minimal protein-encoding gene set are obtained in the course of genome-wide inactivation approaches. The *B. subtilis* approach was based on the insertion of a non-replicating plasmid into the target gene via homologous recombination, and the absence of viable mutants following plasmid transformation indicated gene essentiality under standard laboratory conditions. This assessment was then confirmed by placing the intact gene under control of a regulated promoter, followed by IPTG dependency of the respective strain (Vagner *et al.*, 1998). This approach led to the conclusion that 271 genes of *B. subtilis* are essential, among them only 4% of unknown function.

Transposon mutagenesis

Transposons provide a further method for defining essential nucleic acid sequences. The first transposon-based analysis of a bacterial genome revealed that 55-73% of the *M. genitalium* gene set is essential, and that one third of these genes code for proteins with yet unknown function (Hutchison *et al.*, 1999). A higher efficiency was obtained by applying high-density transposon mutagenesis and genetic footprinting to discrete chromosomal segments of bacterial genomes (Hare *et al.*, 2001; Rubin *et al.*, 1999; Wong and Mekalanos, 2000). Basically, PCR products of 10 kb length were generated from chromosomal DNA as targets for mutagenesis with approximately 5 kb overlap between adjacent fragments. These linear DNA products were then mutagenized by *in vitro* mariner mutagenesis to generate several hundred individual pools of transposon mutants following transformation and homologous recombination. Genetic footprinting was then performed with each mutant to identify chromosomal loci that have not been targeted by transposon insertion, thus indicating the presence of an essential gene (Akerley *et al.*, 1998). The first application of high-density transposon mutagenesis was

reported for *H. influenzae* and detected putative essential roles for the products of 259 yet uncharacterised ORFs corresponding to 54% of all indispensable genes of this organism (Akerley *et al.*, 2002; Reich *et al.*, 1999). Since then, high-density mutagenesis was used to identify essential genes of human pathogens such as *P. aeruginosa* (Jacobs *et al.*, 2003), *H. pylori* (Salama *et al.*, 2004), *E. coli* (Gerdes *et al.*, 2003) or *Mycobacterium tuberculosis* (Lamichhane *et al.*, 2003). However, large-scale analysis of this kind is resource intensive and restricted to naturally competent organisms. As a further limitation, transposon mutagenesis tends to overestimate the set of essential genes by misclassification of nonessential genes that slow down the growth without arresting it, or by missing essential genes that tolerate transposon insertion at a permissive site of an otherwise essential ORF (Table 1). Operon structures are assumed to pose several problems that can be solved only by further analysis. For example, polar effects may reduce the frequency of insertions upstream of an essential gene. In addition, if translation of a downstream essential gene is coupled to a gene that is disrupted by the presence of the insertion, these insertions will not be found (Judson and Mekalanos, 2000b).

In contrast to these “negative” approaches, “positive” transposon mutagenesis identifies essential genes by substitution of the natural promoter of an indispensable gene with an inducible one, generating a conditional mutation. For example, the TnAraOut system uses the arabinose promoter with a large induction ratio. Tn-mutants were replica plated and incubated either with or without arabinose. Colonies which do not grow in the absence of arabinose might have substituted the promoter of an essential gene by the arabinose-induced promoter. Chromosomal DNA of this mutant was isolated, and the targeted region was cloned or amplified via inverse PCR (Judson and Mekalanos, 2000a). However, only insertions upstream of few essential genes were reported, most of them encoding tRNA synthetases with high intracellular copy numbers. This means that expression levels of the applied inducible promoter will not be broad enough to identify a larger set of essential genes.

Insertion-duplication mutagenesis

All approaches discussed above require significant resources to be performed in a high-throughput format. Based on insertional-duplication mutagenesis, a novel screening method that allows the rapid and efficient identification of essential genes within a wide spectrum of bacteria was established. A random fragment library of *S. typhimurium* wild-type strain ATCC 14028 was cloned into a temperature-sensitive vector specific for Gram negative bacteria. A set of 14,000 single *S. typhimurium* clones was then grown under permissive and non-permissive conditions, thus selecting insertional mutants derived from homologous recombination. Clones for which viable insertional mutants were not retained probably contained fragments of essential genes or essential operons. Gene

essentiality was demonstrated for a prioritised set of genes by creating conditionally lethal mutants. The results of this large-scale screening indicated that in rich media, the essential gene set of the *S. typhimurium* wild-type is composed of approximately 490 genes (Knuth *et al.*, 2004). Although 50 bp have been shown to be sufficient for homologous recombination in *Salmonella* (see chapter 3), a limitation of this methodology is that the chosen fragment length of 350-450 bp does not allow to interrupt small genes. Due to the fragment duplication as a result of homologous recombination, the size of the target in which an insertion can occur will generally be small and depends on the gene. Therefore, it might be difficult or impossible to inhibit certain genes.

Many, but not all of the essential genes of *S. typhimurium* had been demonstrated by high-throughput transposon mutagenesis to be indispensable in *E. coli* also (Gerdes *et al.*, 2003). In a more recent attempt to reduce the genome size of *S. typhimurium*, a chromosomal segment of 172 kb could be deleted without affecting cell viability. Within this region, some genes had been proposed to be essential due to the lack of insertional mutants (Nilsson *et al.*, 2005). Several reasons may account for these and other discrepancies, among them genetic differences between the strains used for the studies, the method applied (insertion versus deletion) or differences in growth conditions such as medium compositions, temperature, aeration levels or cell densities (Nilsson *et al.*, 2005). Certain genes may be classified as essential by transposon mutagenesis or insertion-duplication mutagenesis, yet corresponding viable deletion mutants may be obtained in targeted knockout approaches (Gerdes *et al.*, 2003). Table 2 summarizes the results of the most comprehensive studies to define essential bacterial gene sets, most of them overestimating the size of the genome indispensable for cellular growth. Despite the obvious tendency that the number of essential genes increases with the genome size, systematic approaches and *in silico* and theoretical predictions suggest a more compact core of genes absolutely required for cell viability (Castellanos *et al.*, 2004). In line with a very recent suggestion of a robust *Salmonella* metabolism (Becker *et al.*, 2006), a first resume taken from the results described above is that in contrast to former expectations, there is a shortage of novel antibacterial targets among the group of essential genes.

Table 2 (next page): The most comprehensive studies to identify the essential gene sets of pathogenic bacteria or model organisms.

Bacterium	Applied methodology	Genome size [Mb]	Essential genes	Genome portion [%]	reference
Saccharomyces cerevisiae	Systematic gene deletion	12,08	1124	19,0	(Winzeler *et al.*, 1999b)
Pseudomonas aeruginosa	Transposon mutagenesis	6,26	300-400	5,4-7,2	(Jacobs *et al.*, 2003)
Escherichia coli	Transposon mutagenesis	4,64	708	16,6	(Gerdes *et al.*, 2003)
Salmonella typhimurium	Random IDM	4,86	490	11,0	(Knuth *et al.*, 2004)
Mycobacterium tuberculosis	Transposon mutagenesis	4,40	1487	35	(Lamichhane *et al.*, 2003)
Bacillus subtilis	Systematic IDM	4,21	271	6,6	(Kobayashi *et al.*, 2003)
Staphylococcus aureus	Antisense RNA	2,82-2,90	658	~25	(Ji *et al.*, 2001)
Haemophilus influenzae	Transposon mutagenesis	1,83	670	38,0	(Akerley *et al.*, 2002)
Helicobacter pylori	Transposon mutagenesis	1,7	344	23	(Salama *et al.*, 2004)
Mycoplasma genitalium	Transposon mutagenesis	0,58	265-350	55,0-73,0	(Hutchison *et al.*, 1999)
Buchnera spp.	Genome comparison	0,45-0,64	313	50,0-72,0	(Gil *et al.*, 2002)

5. Intracellular metabolism of *Listeria monocytogenes*

(Appendix IV.)

The species *Listeria monocytogenes*

The Gram positive genus *Listeria* comprises six species, two of which, namely *L. ivanovii* and *L. monocytogenes*, are pathogenic. Like *Y. enterocolitica*, *Listeria* spp. are able to grow at refrigeration temperature, making this organism a serious risk for food safety. Domesticated ruminants probably play a key role in the maintenance of *Listeria* spp. in the rural environment, while the foods most often contaminated are dairy products, soft cheeses, smoked fishes and salads. *L. monocytogenes* is responsible for listeriosis, a severe human infection with an overall mortality rate of 30%. The disease is characterized by gastroenteritis, meningitis, encephalitis, abortions and perinatal infections. Despite the ubiquitous distribution of *L. monocytogenes* in soil, water or a variety of foods, the incidence of human listeriosis amounts only to two to eight sporadic cases annually per million people in Europe and the USA (Vazquez-Boland *et al.*, 2001). Central for human listeriosis pathophysiology is the ability of *L. monocytogenes* to cross three tight barriers, namely the intestinal, the blood-brain, and the placental barrier. In addition to these properties, *L. monocytogenes* is a facultative intracellular parasite able to infect and multiply within macrophages and non-phagocytic cells, such as epithelial cells, fibroblasts, and hepatocytes.

Intracellular lifestyle

The intracellular cycle of *L. monocytogenes* starts with adhesion to the surface of the eukaryotic cell via the internalins and subsequent invasion into the host cell by the so-called “zipper” mechanism during which the bacteria are engulfed within a phagocytic vacuole that becomes acidified soon after uptake. *L. monocytogenes* probably prevents phagosome maturation to the phagolysosomal stage, a defence strategy that is also characteristic of *Salmonella*. In contrast to this intracellular pathogen, *L. monocytogenes* is capable to escape from the intracellular vacuole by disrupting the phagosomal membrane. A cytolysin belonging to the family of cholesterol-dependent, pore-forming toxins with a low pH optimum of 5.5 was identified to be responsible for this key step in listerial pathogenicity. Following lysis of the vacuole, cytosolic *Listeria* induces polymerization of actin filaments that promote bacterial intracellular movement; a single bacterial factor, the surface protein ActA, is responsible for this actin-based motility. Thus, *Listeria* is able to directly spread to neighbouring epithelial cells where the cycle restarts. This is reflected by the ability of *L. monocytogenes* to form plaques in monolayers of tissue-culture cells. Within the cytosol, the *Listeria* cells replicate with a doubling time of approximately 1 h, i. e. only three times slower than in rich medium

indicating that the intracellular compartment is permissive for efficient bacterial proliferation (Vazquez-Boland *et al.*, 2001). However, little is known about the intracellular availability of nutrients essential for bacteria, about the metabolism of *L. monocytogenes* during replication in the cytosol, and about specific genetic determinants that help this pathogen to proliferate within host cells, thus increasing the infective load within its host. A better understanding of the intracellular replication strategies of *L. monocytogenes* might give rise to the identification of potential antilisterial targets.

A system analogous to IVET used the listeriolysin gene, *hly*, as a reporter to identify listerial genes induced *in vivo* (Gahan and Hill, 2000). In this screen, a mutant of fumarate hydratase was identified and shown to be attenuated in the mouse model and in cultured phagocytes. Another protein of *L. monocytogenes*, Hpt, involved in the exploitation of hexose phosphate from the host cell, was only recently shown to be the first virulence factor specifically involved in intracellular replication (Chico-Calero *et al.*, 2002). Hpt expression is tightly controlled by the master virulence regulator PrfA that determines the expression of at least 73 genes as shown by whole-genome expression profiling (Milohanic *et al.*, 2003). In addition, a *L. monocytogenes* mutant lacking the lipoate protein ligase LplA1 was defective for growth specifically in the host cytosol (O'Riordan *et al.*, 2003). The E2 subunit of pyruvate dehydrogenase (PDH) was demonstrated to lack a critical lipoyl modification in a Δ*lplA*1 strain, indicating that the avirulent phenotype and abortion of intracellular growth was due to the loss of PDH function.

Systematic analysis of *L. monocytogenes* replication in eukaryotic cells

So far, the listerial genome has not systematically been investigated for genes involved in cytosolic replication. For that purpose, a mutant library of *L. monocytogenes* consisting of approximately 760 strains was investigated for their ability to replicate in Caco-2 cells (Fig. 3). Although tissue-culture systems are artificial systems and therefore lack the complexity of the human body, they should mimic the subtle interactions that occur during cytoplasmic replication of intracellular pathogens. For a highly invasive pathogen such as *L. monocytogenes*, a proven correlation exists between data obtained in cell culture experiments and the true situation *in vivo*, also indicating that there is an intracellular phase during infection (Miller, 1995). Thus, qualitative cell culture assays will contribute to the understanding of the nature of intracellular environments.

The 760 mutants tested in the gentamycin protection assay were calculated to represent approximately 16% of all 2853 listerial genes. Mutants exhibiting growth deficiencies in BHI medium were excluded from the screening procedure. Of all mutants, only one carrying a plasmid insertion into *inlA* encoding internalin A, was severely affected in its adhesion or invasion capability. By reverting 15 insertion mutants to the wild-type

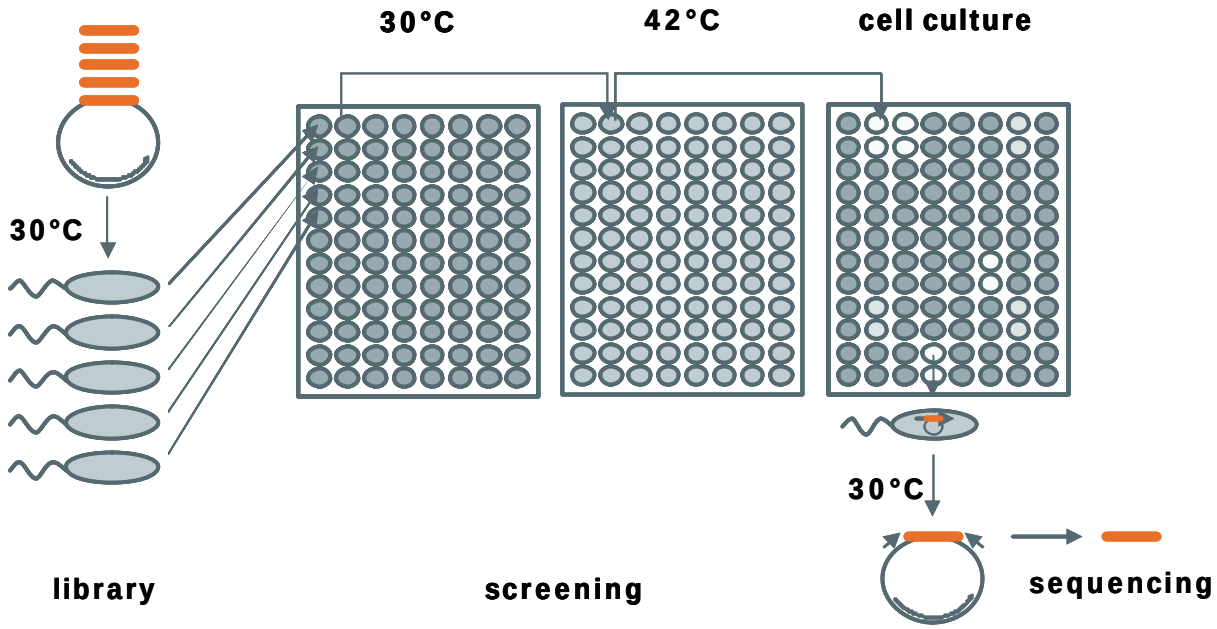

Fig. 3: Strategy to identify listerial genes involved in intracellular replication. Short, random chromosomal fragments of the *L. monocytogenes* chromosome were cloned into the temperature-sensitive plasmid pLSV101. The resulting fragment library was transformed into *L. monocytogenes*, and insertion mutants were selected upon shift to non-permissive temperature. The mutant library was then tested for growth deficiencies in Caco-2 cells, and attenuated mutants were genetically characterized.

phenotype, it was confirmed that the observed growth attenuation in epithelial cells is not due to independent mutations, but to the site-specific insertion. A total of 72 mutants fulfilled all validation criteria, thus representing a group of genes that are important for intracellular replication of *L. monocytogenes* (Joseph *et al.*, 2006). A classification of these genes according to their known or predicted cellular function is shown in Fig. 4.

Insertional mutagenesis might induce polar effects, resulting in modified expression of genes located downstream of the insertion site. Many of the mutants identified are monocistronically transcribed according to the genome annotation of *L. monocytogenes*. In several cases, the plasmid insertion affected genes organized in operons of which all genes are probably involved in the same cellular function. Polar effects could be excluded for other mutants due to terminators located immediately downstream of the insertion. Real-time RT-PCR revealed that an insertion of the temperature-sensitive vector into gene lmo1971 does not significantly affect the transcription of a gene organized within the same operon. A reason for this observation is that the plasmid carries outward directed promoters that drive the transcription of downstream located genes, and that polar effects are therefore relatively rare.

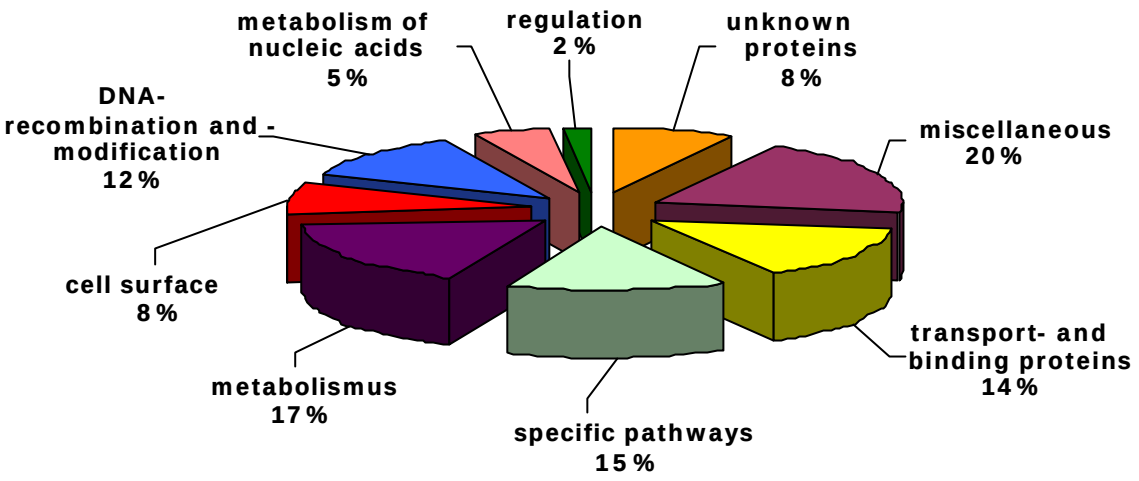

Fig. 4: Classification of listerial genes that were identified to play a role in intracellular survival. A majority of genes belongs to the categories transport, metabolism and specific pathways. For only 8% of the gene products, no homologies to other bacterial proteins could be found.

Intracellular requirements of *L. monocytogenes*

L. monocytogenes is a multiple-amino-acid auxotrophic species that requires aromatic amino acids or purines for growth in minimal medium. Thus, adaptation to intracellular parasitism by this microbial pathogen involves exploitation of metabolic products of its host cell. In general, intracellular proliferation of this pathogen indicates that the cytoplasmic compartment provides sufficient organic and inorganic compounds to overcome these auxotrophies. Since *L. monocytogenes* is unable to hydrolyze proteins, its growth in nutrient-limited compartments such as host cells depends upon physiological adaptation that allows usage of nutrients provided by the environment. The uptake of peptides and free amino acids usually involves binding-protein-dependent permeases which are members of the ATP-binding cassette (ABC) transporter superfamily. Among these transporters are the oligopeptide permease (Opp) systems involved in nutrient uptake and cell wall peptide recycling. *L. monocytogenes* OppA has been shown to be required for bacterial growth both at low temperature and in macrophages (Borezee *et al.*, 2000). During the described screening, at least 11 further transporters have been shown to play an important role in intracellular replication of *L. monocytogenes* (Fig. 4).

A remarkable result of the above described screening was the identification of a species-specific gene cluster (lmo1968-1974) that encodes a pentitol-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS). An intracellular survival rate of approximately 50% with respect to the wild-type was observed for a non-

polar deletion mutant of this putative operon. However, the biological role of this transporter during intracellular replication still remains to be disclosed.

The reduced growth of strains with insertion or deletion mutations of lmo1538, encoding a glycerol kinase, and of *glpD* (lmo1293) that encodes a glycerol-3-phosphate-dehydrogenase, suggests that glycerol plays a role as a carbon source for listerial growth in epithelial cells. Interestingly, the insertional knockout of *eutB* involved in ethanolamine utilization also led to reduced intracellular replication. Ethanolamine, together with glycerol, might be the result of degradation of phosphatidylethanolamine derived from phospholipids of host cell membranes. Ethanolamine may then be converted by the coenzyme B_{12} dependent activity of EutB and EutC to acetaldehyde and ammonia. This model of additional carbon and nitrogen sources exploited by intracellular listeriae is confirmed by the observation that beside the genes mentioned, lmo1034 encoding another glycerol kinase, several genes involved in coenzyme B_{12} synthesis and the *eutABC* operon are significantly up-regulated during cytosolic proliferation (Joseph *et al.*, 2006). Taking into account similar results obtained for *S. typhimurium* (Fuchs, unpublished data), these findings provides evidence that coenzyme B_{12} dependent activities play an important role in intracellular growth of pathogens and probably also in their virulence properties. A threefold attenuation of an *ilvD* mutant indicates the importance of amino acids that are synthesized by *L. monocytogenes* and other facultative intracellular pathogens, but not by the mammalian host cells. This assumption is in line with the upregulation of genes involved in the biosynthesis of branched-chain amino acids, namely leucin, isoleucin, and valin, and of tryptophan and arginine.

Microarray analysis is poorly predictive for biological relevant functions

In two DNA microarray studies performed independently, it was found that approximately 17-19% of all genes of *L. monocytogenes* are differentially regulated during intracellular growth (Chatterjee *et al.*, 2006; Joseph *et al.*, 2006). Evidence was provided that the pentose phosphate cycle, rather than glycolysis, is the predominant pathway of sugar metabolism of *L. monocytogenes* following transition from the extracellular milieu to the cytoplasm of the infected host cell.

The ratio behind transcriptome analysis is that differential gene induction correlates with a biological function under the applied conditions. However, interfacing and extrapolation of the results of mutant screening and microarray data revealed that only approximately 36% of all up-regulated genes are indeed required for listerial proliferation in the cytosol of epithelial cells. This data indicates that DNA microarray analysis is poorly predictive of pathways required for intracellular replication. A similar observation has only recently been reported for *M. tuberculosis* survival in macrophages (Chatterjee *et al.*, 2006).

6. Association of *Yersinia enterocolitica* with invertebrates

(Appendix I.-III.)

The species *Yersinia enterocolitica*

The genus *Yersinia* is composed of 11 species, three of which (*Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) have clearly been shown to be pathogenic for humans. The *Yersinia* species are Gram-negative coccobacilli which belong to the familiy of Enterobacteriaceae. *Y. enterocolitica* is an important gastrointestinal pathogen which causes a range of human diseases such as mild diarrhoea or mesenteric lymphadenitis (Bottone, 1997). The presence of *Y. enterocolitica* in food products is of particular concern since it is capable to survive and grow at refrigeration temperatures and even at -5°C (Pritchard, 1994). This psychrotolerant pathogen can be isolated from a wide variety of sources such as surface water, seafood, dairy products and livestock, with swine serving as a major reservoir for human pathogenic strains. Most non-porcine isolates of *Y. enterocolitica* are frequently isolated from terrestrial and freshwater ecosystems and belong to the non-pathogenic biovar 1A, probably due to the lack of the virulence determinants (Bottone, 1999). In contrast, all strains of biovars 2-5 are obligate pathogens for humans and domestic animals and carry the virulence plasmid pYV. Biovar 1B strains commonly isolated from patients in the USA form a geographically distinct group, and are particularly virulent for humans due to a so-called “high-pathogenicity island”, HPI (Schubert *et al.*, 2004).

Given that the usual route of *Y. enterocolitica* acquisition is through contaminated food, this bacterium must undergo a temperature adaptation for a successful colonization of the mammalian host tissue. To achieve this stage of pathogenesis, *Y. enterocolitica* makes use of temperature-dependent virulence determinants that are maximally expressed at 37°C, such as Ail and YadA involved in cell attachment or the Yop proteins that play a role in circumventing host defense mechanisms. Moreover, the invasin Inv which promotes cell penetration is maximally expressed at temperatures below 28°C, resulting in cells primed for early epithelial cell invasion following oral uptake from a cold reservoir. A further example for temperature-regulated determinants are the flagellar genes that are repressed at 37°C, but induced at lower temperature. Their role in pathogenesis of Yersiniae, however, is yet unclear.

The association between temperature-dependent gene regulation and adaptation to the host organism has long been established for this bacterial pathogen. It gave rise to the concept of a biphasic life cycle of *Y. enterocolitica* during which this pathogen encounters at least two distinct ambient conditions characterized by 37°C in the mammalian body and the varying environmental temperature (Straley and Perry, 1995). However, very little is known about the molecular mechanisms that allow yersiniae a long-term

adaptation to low temperatures. To better understand the behaviour of a pathogen outside the human host, the genome of *Y. enterocolitica* was systematically investigated for chromosomal factors that play an important role in its survival and propagation in environmental reservoirs.

Expression profile at low temperature

A hallmark of bacteria is there adaptability to environmental conditions. Many microbial studies of differential gene expression in response to alterations in ambient conditions have been performed, but were restricted to the characterisation of few target loci only. In the genomic era, novel tools are required to probe microbial behaviour in a more comprehensive manner. DNA microarray studies yield a snapshot of the global response of a microorganism at a defined time point only, and do not allow to track gene expression in a real-time fashion. Moreover, to prove the biological relevance of observed transcriptional changes, further experimental efforts such as mutational analysis are required. So far under-used reporter gene technologies based on bioluminescence are able to overcome such limitations (Rhodius *et al.*, 2002; Van Dyk *et al.*, 2001b). The use of promoter fusions to the bioluminescent *luxCDABE* genes provides an independent method compared to hybridization experiments as demonstrated by analysis of *E. coli* gene responses to DNA damages (Van Dyk *et al.*, 2001a) and several other applications (Qazi *et al.*, 2004). Since the *luxCDABE* system produces light without the addition of exogenous substrate, it is non-invasive and enables efficient transcriptional profiling at any time point, as well as facile repetition of the measurement of promoter activities. A striking advantage of the luciferase system over the well established GFP reporter is its high sensitivity that enables the detection of transcriptional signals over a dynamic range of at least five orders of magnitude, and thus also the identification of down-regulated genes (Van Dyk, 2002).

While the pathogenic determinants of *Y. enterocolitica* involved in causing disease have been investigated in detail, little is known about the molecular mechanisms that enable this species to survive and proliferate in non-mammalian environments. As a first step in functional genomic studies with *Y. enterocolitica*, a random transposon mutant library of approximately 5700 clones was established using a suicide plasmid that harbours a Tn*5* transposon and a promoterless copy of the luminescence reporter genes *luxCDABE*. The continuous expression profile of all mutants at 30°C and 10°C, measured as relative light units per culture density, was derived, and a series of genes strongly induced during prolonged growth at low temperature was identified (Bresolin *et al.*, 2006b).

Y. enterocolitica gene induction beyond cold acclimatization

A psychrotolerant microorganism such as *Y. enterocolitica* requires specific adaptive strategies in order to overcome challenges faced at low temperatures, such as decrease in membrane fluidity, increase in negative supercoiling of DNA and inhibition of translational initiation of bulk mRNA (Stübs *et al.*, 2005). None of the investigated transposon insertions affected a gene or gene complex that is dispensable for cell growth at 30°C, but required for growth at low temperature, such as PNPase (Goverde *et al.*, 1998), indicating that long-term adaptation to cold is rather the result of a number of adaptive processes than the activity of a single factor or regulon. The pronounced separation between shock response and long-term adaptation revealed to be a general feature of bacterial gene expression induced by environmental stress. After leaving the host and, as an example, entering an aquatic habitat, *Y. enterocolitica* experiences a drastic temperature drop and serious nutrient limitations through dilution. The specific expression profile at low temperature, as discussed below, strongly supports the view of temperature as an important stimulus for the long-term response of *Y. enterocolitica* to such a changing environment. Roughly, the genes induced at low temperature can be grouped into three categories according to the temporal order of expression, thus indicating that the long-term response of this pathogen to cold is a multistage developmental program.

i) The initial response of bacteria to environmental changes like temperature decrease is mediated by *sensory proteins and regulatory networks.* Signal transduction mechanisms allow bacteria to know where they are and to express different sets of genes within and outside the host. It is therefore not surprising to observe the induction of several regulatory or signal transduction systems of known (ArcA, UhpABC) and predicted function when *Yersinia* cells are exposed to cold. The ArcAB system, for example, controls the expression of at least 40 operons involved in catabolic gene expression (Alexeeva *et al.*, 2003; Sevcik *et al.*, 2001).

ii) The most prominent response of *Y. enterocolitica* to low temperature is the induction of genes involved in *chemotaxis and motility*, while cells are immobile during growth at 37°C due to repressed transcription of *Y. enterocolitica* flagellin-encoding genes *fleABC* (Kapatral *et al.*, 1996). The subsequent induction of proteins involved in chemotaxis enables to respond to attractants, and it might therefore be speculated that enhanced motility will allow the pathogen to open new nutrition sources in the environment.

iii) *Metabolic pathways* are activated in *Y. enterocolitica* at 10°C before entering stationary phase. Appropriate expression of metabolic enzymes during late exponential growth phase might be crucial for survival within the stationary phase. During late growth of *Y. enterocolitica*, the expression of histidine ammonia-lyase with homology to the HutH histidase involved in nitrogen utilization, and of the α-glucan branching enzyme GlgB is strongly induced. The latter one, together with the α-amylase GlgX and GlgC,

regulates the glycogen metabolism (Romeo *et al.*, 1988). Interestingly, the biodegradative *hut* operon as well as an alpha-amylase have been shown to be up-regulated at low temperature in several antarctic psychrotrophic bacteria (Feller *et al.*, 1992; Kannan *et al.*, 1998). In addition, a hot spot of mini-Tn*5 luxCDABE* insertions has been found in a gene encoding an alkaline serine protease, a member of the extracellular subtilisin family (Valbuzzi *et al.*, 1999).

Insecticidal activity of *Y. enterocolitica*

One of the most intriguing results of this study was the identification of two transposon insertions within the so-called toxin complex (*tc*) genes that had not yet been found in the genome of any *Y. enterocolitica* strain. Tc homologues have been described in yersiniae and in other insect-associated bacteria such as *Serratia entomophila*, *Xenorhabdus nematophilus* or *Photorhabdus luminescens* (Waterfield *et al.*, 2001b). By expressing a variety of virulence factors including the Tc proteins, the bacteria are suggested to help in killing the insect host, thus providing a source of nutrients. The Tc proteins were first purified from *P. luminescens* (Bowen and Ensign, 1998). The respective genes encode four high molecular weight toxin complexes that are termed Tca, Tcb, Tcc and Tcd whose oral insecticidal activities are comparable to those of *Bacillus thuringiensis* toxins. Studies based on recombinant expression of *tc* genes from *P. luminescens* in *E. coli* showed that the combination of three genes, *tcdA*, *tcdB*, and *tccC*, is essential for oral toxicity towards *M. sexta* (Waterfield *et al.*, 2001a). Further experiments supported the hypothesis that TccC-like proteins might act as universal activators of, or chaperons for, different toxin proteins, while Tca-like and Tcd-like proteins contribute predominately to the oral toxicity of bacterial supernatants (ffrench-Constant *et al.*, 2003). The host spectra vary among insecticidal bacteria investigated so far. For example, *P. luminescens* exhibits a strong toxic activity when fed to the larvae of *M. sexta*, while *X. nematophilus* is lethal for the larvae of *Pieris brassicae*. Comparison of the putative enzymatically active Tc proteins from *Y. enterocolitica*, *Y. pestis*, and *Y. enterocolitica* revealed a high variability in their amino acid sequences (21% to 71%), suggesting a broad host spectrum which *Y. enterocolitica* is adapted to (Bresolin *et al.*, 2006a). Five *tc* genes of *Y. enterocolitica* were shown to be located on a chromosomal 19-kb fragment termed *tc* pathogenicity island (*tc*-PAI^{Ye}, Fig. 5). Similar islands are also present in the genomes of other two human-pathogenic *Yersinia* species, namely *Y. pestis* and *Y. pseudotuberculosis*. Like many other pathogenicity islands, *tc*-PAI^{Ye} displays a different overall base composition from the core chromosomal elements, suggesting that it was acquired from a foreign source (Hacker *et al.*, 1997).

A *lux*-reporter mutant grown at 10°C to 37°C revealed an maximal expression of *tc*-genes at 10°C during late logarithmic and early stationary phase, while no activity in terms of bioluminescence above background was observed at 37°C, indicating a complete

repression of insecticidal genes at mammalian body temperature (Bresolin *et al.*, 2006a). In comparison to 37°C, the transcriptional activity of the reporter genes increased by more than four orders of magnitude when the growth temperature declined to 10°C-20°C. Once those strain-specific genes are identified, however, the challenge remains to devise ways to study their biological functions. To this end, whole-cell extracts of *Y. enterocolitica* cultivated at 30°C and 15°C where tested for their insecticidal potential. It could be demonstrated that extracts from cells grown at low temperature, but not at mammalian body temperature, were orally active against the larvae of *M. sexta*, the tobacco hornworm (Bresolin *et al.*, 2006a). In contrast, a deletion mutant of *tcaA* failed to display toxic activity against insect larvae, clearly indicating that *tcaA* encodes a protein responsible for killing the insect host. This was the first demonstration that *tc* genes of *Yersinia* spp. are biologically active, giving rise to speculations about a yet undisclosed niche of *Y. enterocolitica* in insects.

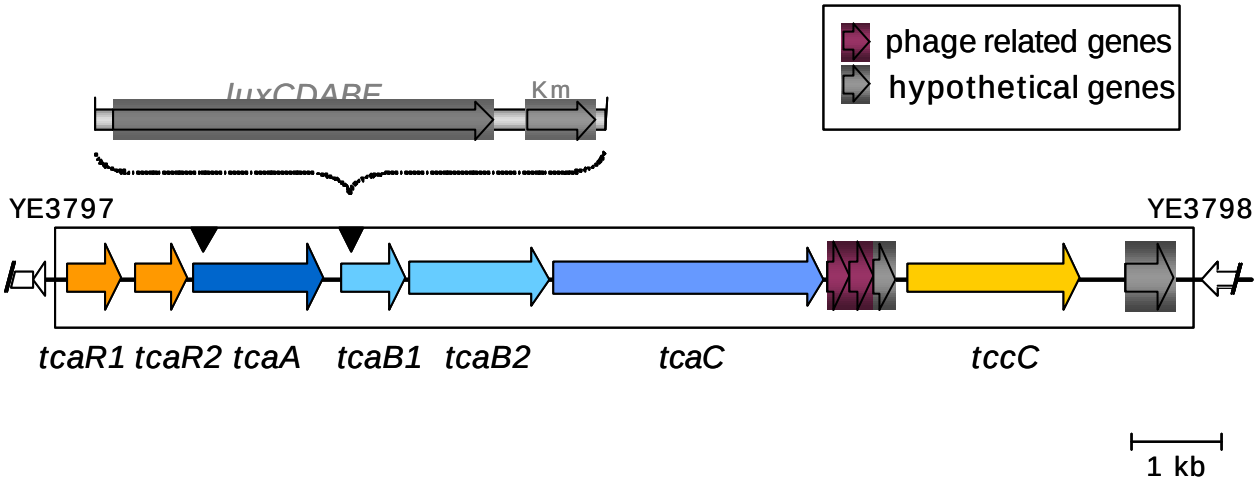

Fig. 5: Genetic organization of the insecticidal pathogenicity island *tc*-PAI^{Ye} of *Y. enterocolitica*. The triangles indicate chromosomal insertions of the luciferase reporter cassette that led to the detection of low-temperature induced toxin complex genes.

Invertebrates as hosts for *Y. enterocolitica*?

Recently, insecticidal Tc homologues have been suggested to be important in the association of *Y. pestis* and *Y. pseudotuberculosis* with insects (Waterfield *et al.*, 2004). *Y. pestis*, a blood-borne pathogen and the etiological agent of human plague, has long been known to be transmitted by insects, specifically by fleas. Using *Caenorhabditis elegans* as a model, it was shown that *Y. pestis* creates an *hmsHFRS*-dependent extracellular biofilm as a mechanism to prevent predation and to permit flea-borne transmission (Darby *et al.*, 2002; Joshua *et al.*, 2003). Due to the absence of *hmsHFRS*, such a blockage effect was not observed for *Y. enterocolitica*, thus allowing the uptake by the nematode. Recent experimental data, as well as genome comparison, provide

evidence that additional genetic determinants beside the *tc* genes are involved in the association of *Y. enterocolitica* with insects. For example, the transcription of two other putative virulence factors, a homologue of the SsrAB-activated SrfA (Worley *et al.*, 2000), and a homologue of FhaC from *B. pertussis* involved in hemolysin activation, was shown to be low temperature responsive, suggesting a role of these two putative virulence determinants outside mammalian hosts. At least two virulence factors of *Y. enterocolitica* have been known to be maximally expressed at ambient temperatures, the heat-stable enterotoxin (Boyce *et al.*, 1979) and the outer-membrane protein Inv (Pepe *et al.*, 1994). In an attempt to disclose a regulatory network among the set of genes induced at low temperature, an inhibitory effect of *uhpC* and one of the chemotaxis operons on the transcription of the FhaC-like transporter was observed. A similar phenomenon was reported recently for expression and activity of a *X. nematophilus* hemolysin that is required for full virulence against insects (Cowles and Goodrich-Blair, 2005). Interestingly, microarray data of *S. typhimurium* motility only recently revealed the surface-dependent regulation of several virulence genes, among them *srfABC*. These interdependencies confirm the linkage between motility and virulence properties at the level of regulation that is observed in many bacteria (DiRita *et al.*, 2000; Harshey, 2003; Wang *et al.*, 2004). None of the above mentioned genes involved in degradative metabolism is expressed at 37°C (data not shown), again suggesting a role of these enzymes outside a mammalian host. For example, the elevated expression of a subtilisin-like protease might play a role in the bioconversion of the insect host attacked by the *tc* gene products.

The temperature-dependent expression of insecticidal *Yersinia* genes indicated that the association with insects, possibly in a mutualistic relationship with nematodes, is indeed an important stage in the lifecycle of *Y. enterocolitica*, and that *Y. enterocolitica* can switch between pathogenic and apathogenic phases. Temperature and nutrient availability seem to be critical parameters for the repression or induction of genes correlated with these phases, among them virulence factors directed against mammalian or invertebrate cells (Fig. 6). The investigations described led to the assumption that poikilothermic insects are a yet overseen reservoir of *Y. enterocolitica*, and that the *tc*-PAI^{Ye} and others, yet uncharacterized factors plays an important role in the transmission and survival of pathogenic *Y. enterocolitica* strains outside mammalian hosts. This model not only provides new insights into the ecology and the life cycle of yersiniae, but is also relevant for strategies to prevent transmission of these pathogens and demonstrates the potential of the *Yersinia* toxin complex as an alternative to the insecticidal toxin of *B. thuringiensis* (Bresolin *et al.*, 2006c).

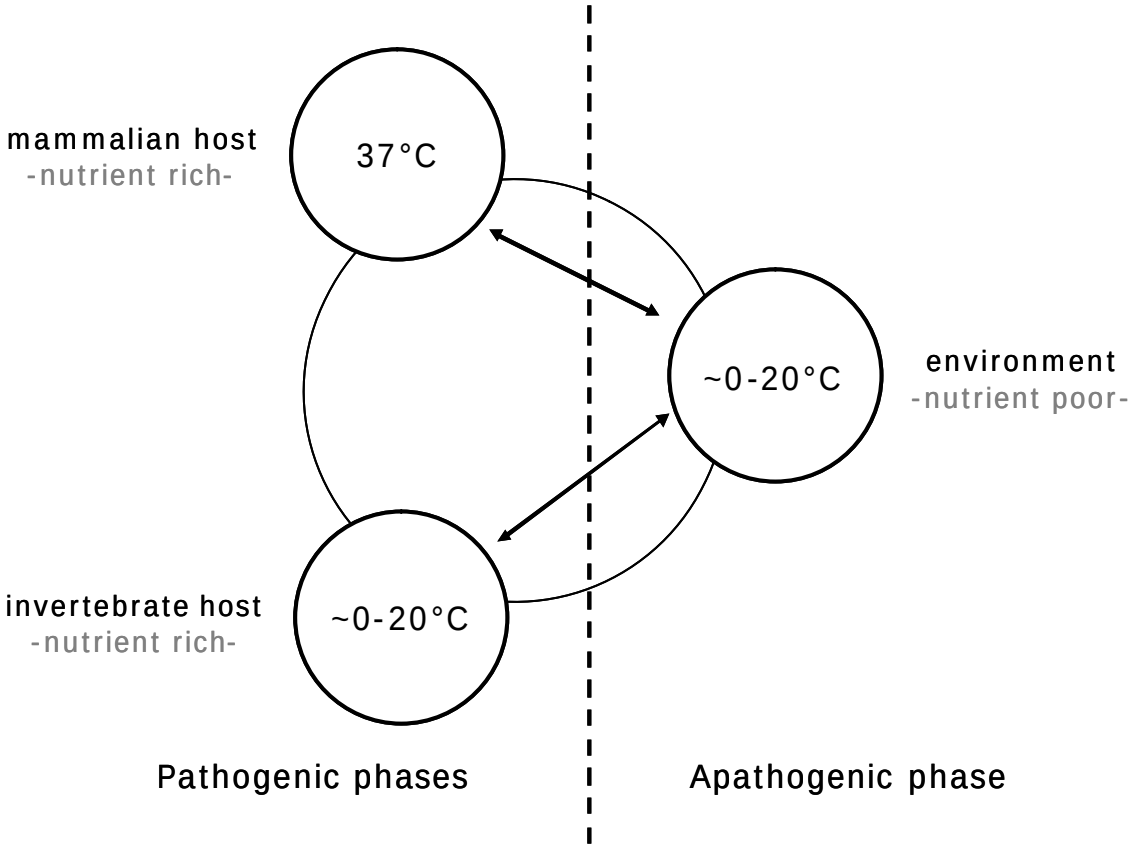

Fig. 6: Hypothetical and simplified model of the *Y. enterocolitica* life-cycle as suggested by the presented data. Different sets of putative or characterized virulence factors are expressed in two distinct pathogenic phases. The lack of nutrition factors as encountered in the environment may play a role in the repression of pathogenicity factors during growth of *Y. enterocolitica* in the apathogenic phase.

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9. Author's publications and patents

A) Publications

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Middendorf, B, Stübs, D., Guiso, N., Deppisch, H., Gross, R, and T. M. Fuchs (2005). Phg, a novel member of the autotransporter family present in *Bordetella* species. *Microbiological Research* 160, 329-336.

Fuchs, T. M., Klumpp, J., and K. Przybilla (2005). Insertion-duplication mutagenesis (IDM) of *Salmonella enterica* and related species using a novel thermosensitive vector. *Plasmid* 55, 39-49.

Bresolin, G., Morgan, A., Ilgen, D., Scherer, S., and T. M. Fuchs (2006): Low temperature-induced insecticidal activity of *Yersinia enterocolitica*. *Mol. Microbiol.* 59, 503-512.

Joseph, B., Przybilla, K., Stühler, C., Schauer, K., Slaghius, J., Fuchs, T. M., and W. Goebel (2006). Identification of *Listeria monocytogenes* genes contributing to intracellular replication by expression profiling and mutant screening. *J. Bacteriol.* 188, 556-568.

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B) Patents

Hueck, C. J., Apfel, H., Fuchs, T. M., Gibbs, C. P., and T. F. Meyer (2000) Essential genes and gene products for identifying, developing and optimising immunological and pharmacological active ingredients for the treatment of microbial infections. Applicant: Max Planck Gesellschaft; CREATOGEN AG. Application number: CA20002385822 20000531.

Fuchs, T. M. (2002) Screening for antimicrobial drug targets by genome-saturating mutagenesis (GSM) Applicant: Fuchs, T. M.; CREATOGEN AG. Application number: WO2002EP03874 20020408.

Apfel, H., Fuchs, T. M., Freissler, E., and H. S. Niesalla (2002) Screening method for attenuating or virulence defective microbial cells. Applicant: Apfel, H.; Fuchs, T. M.; CREATOGEN AG; Freissler, E.; Niesalla, H. S. Application number: WO2002EP05493 20020517.

Bresolin, G., Scherer, S., and T. M. Fuchs (2005). Verwendung bakterieller insektizider Toxine. Applicant: T. M. Fuchs. Submitted to the Deutsches Patent- und Markenamt. Priority date (DPMA): 23.11.2005. Akz: 10 2005 055 689.2.

10. Curriculum vitae

Name:	Thilo Martin Fuchs
Private address:	Schloss Oberellenbach 84066 Mallersdorf-Pfaffenberg Germany Tel.: 0049- (0) 8772-804147
Academic degrees:	Dr. rer. nat., Diploma in Biology

Professional career

Since April 2004:	Habilitand at the Wissenschaftszentrum Weihenstephan
Since Sept. 2002:	Senior scientist and group leader at the ZIEL, Department of Microbiology, Technical University Munich, Germany
Jan. 1999-May 2002:	Post-doc scientist and group leader at CREATOGEN AG, Augsburg, Germany
Aug. 1998-Dec. 1998:	Post-doc scientist in the Helicobacter Vaccine Project at the Max-Planck-Institute for the Biology of Infections, Tübingen, Germany
Sept. 1997-July 1998:	Scholarship from the Deutsche Forschungsgemeinschaft (DFG) at the Department of Microbiology, University of Würzburg, Germany
July 1996-Aug. 1997:	Post-doc scientist at the Department of Microbiology, University of Würzburg
July 1996:	Ph. D. graduation (“magna cum laude”)
March 1992-June 1996:	Ph. D. student at the Theodor-Boveri-Institute, Department of Microbiology (chair: W. Goebel), University of Würzburg
Dec. 1994-May 1995:	Research stay at the Istituto di Ricerche Immunobiologiche di Siena, Italy, (director: R. Rappuoli)
Oct. 1991-Febr. 1992:	Assistant scientist at the Institute for Physiology, University of Würzburg
March 1991-Sept. 1991:	Bacterial Diagnostic

National service

March 1990-Febr. 1991:	Assistant male nurse at the Nephrology Department of the Luitpold-hospital, University of Würzburg

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Higher education

Febr. 1990:	Diploma-examination (M. Sc.) of the faculty of Biology, University of Würzburg
Jan. 1989-Febr. 1990:	Employed as undergraduate laboratory scientist whilst working for the Diploma-thesis
April 1987:	Intermediate examinations in Biology
April 1986-Febr. 1990:	Continued with Biology at the University of Würzburg
April. 1985-March 1986:	Took two semesters out to study Philosophy and Physics at the University of Bielefeld, Germany
Oct. 1983-July 1984:	Started my Diploma in Biology at the University of Würzburg

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Schools

May 1983:	A-levels (Abitur) in Mathematics, German, Biology and History
1974-1983:	Rabanus-Maurus-School (grammar school) in Fulda, Germany
1970-1974:	Elementary school in Fulda

Further academic education

2006	PROLEHRE compact course, Carl von Linde academy, Technical University of Munich
2004	Intermediate examinations in economics, University of Hagen, Germany
2001	Certificated knowledge of economy, IWW-institute of the University of Hagen
2000	Project management, academy of Sueba, Germany

11. Appendix: selected publications

I. Bresolin, G., Morgan, A., Ilgen, D., Scherer, S., and T. M. Fuchs (2006): Low temperature-induced insecticidal activity of *Yersinia enterocolitica*. ***Mol. Microbiol.*** **59**, 503-512.

II. Bresolin, G., Neuhaus, K., Scherer, S., and T. M. Fuchs (2006). Transcriptional analysis of long-term adaptation of *Yersinia enterocolitica* to low temperature growth. ***J. Bacteriol.*** 188, 2945-2958.

III. Stübs, D., Fuchs, T. M., Schneider, B., Bosserhoff, A., and R. Gross (2005). Identification and regulation of cold inducible factors of *Bordetella bronchiseptica*. ***Microbiology*** **151**, 1911-1917.

IV. Joseph, B., Przybilla, K., Stühler, C., Schauer, K., Slaghius, J., Fuchs, T. M., and W. Goebel (2006). Interfacing expression profiling and mutant screening: identification of *Listeria monocytogenes* genes that contribute to intracellular replication. ***J. Bacteriol.*** **188**, 556-568.

V. Knuth, K., Niesalla, H., Hueck, C. J., and T. M. Fuchs (2004). Large-scale identification of essential *Salmonella* genes by trapping lethal insertions. ***Mol. Microbiol.*** **51**, 1729-1744.

VI. Fuchs, T. M. (2005). Reduzierte Genome und das Konzept eines minimalen Gensets. ***GenomXpress*** **01**, 22-24.

VII. Fuchs, T. M., Klumpp, J., and K. Przybilla (2005). Insertion-duplication mutagenesis (IDM) of *Salmonella enterica* and related species using a novel thermosensitive vector. ***Plasmid*** **55**, 39-49.

VIII. Fuchs, T. M., Schneider, B., Krumbach, K., Eggeling, L., and R. Gross (2000). Characterization of a *Bordetella pertussis* diaminopimelate (DAP) biosynthesis locus identifies *dapC*, a novel gene coding for an N-succinyl-L,L-DAP aminotransferase. ***J. Bacteriol.*** **182**: 3626-3631.

IX. Middendorf, B, Stübs, D., Guiso, N., Deppisch, H., Gross, R., and T. M. Fuchs (2005). Phg, a novel member of the autotransporter family present in *Bordetella* species. ***Microbiological Research*** **160**, 329-336.

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