Large-Scale Identification of Pathogenicity Factors in *Bartonella* by Signature-Tagged Mutagenesis

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Luis-Henri Saenz Dürrmeier

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auf Antrag von

Prof. Christoph Dehio

Prof. Urs Jenal

Prof. Guy Cornelis

(Dissertationskomitee)

Basel, den 05.04.2005

Dekan: Prof. Hans-Jakob Wirz

FÜR SIMONE

STATEMENT TO MY PHD THESIS

This work was carried out from March 2001 to April 2005 in the group of Prof. Christoph Dehio in the Division of Molecular Microbiology at the Biozentrum of the University of Basel. My PhD thesis committee consisted of:

Prof. Christoph Dehio

Prof. Urs Jenal

Prof. Guy Cornelis

My PhD thesis is written in a cumulative format. It consists of the published review article about the signature-tagged mutagenesis method used for my work and the manuscript of a research article about my work. The chapter “Concluding Remarks” summarizes the major findings, the chapter “Perspective” discusses the continuation of the projects started based on my work.

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ELSEVIER

Signature-tagged mutagenesis: technical advances in a negative selection method for virulence gene identification

Henri L Saenz and Christoph Dehio

Signature-tagged mutagenesis (STM) is a powerful negative selection method, predominantly used to identify the genes of a pathogen that are required for the successful colonization of an animal host. Since its first description a decade ago, STM has been applied to screen a vast amount of transposon insertion mutants in 31 bacterial species. This has led to the identification of over 1700 bacterial genes that are involved in virulence. Despite the preservation of the basic design, the STM method has been developed further owing to recent advances including different designs of the signature-tags and profound changes in the mode of detection. These advances promoted substantially the application range and versatility of the STM method.

Addresses
Division of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

Corresponding author: Dehio, Christoph (christoph.dehio@unibas.ch)

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Introduction

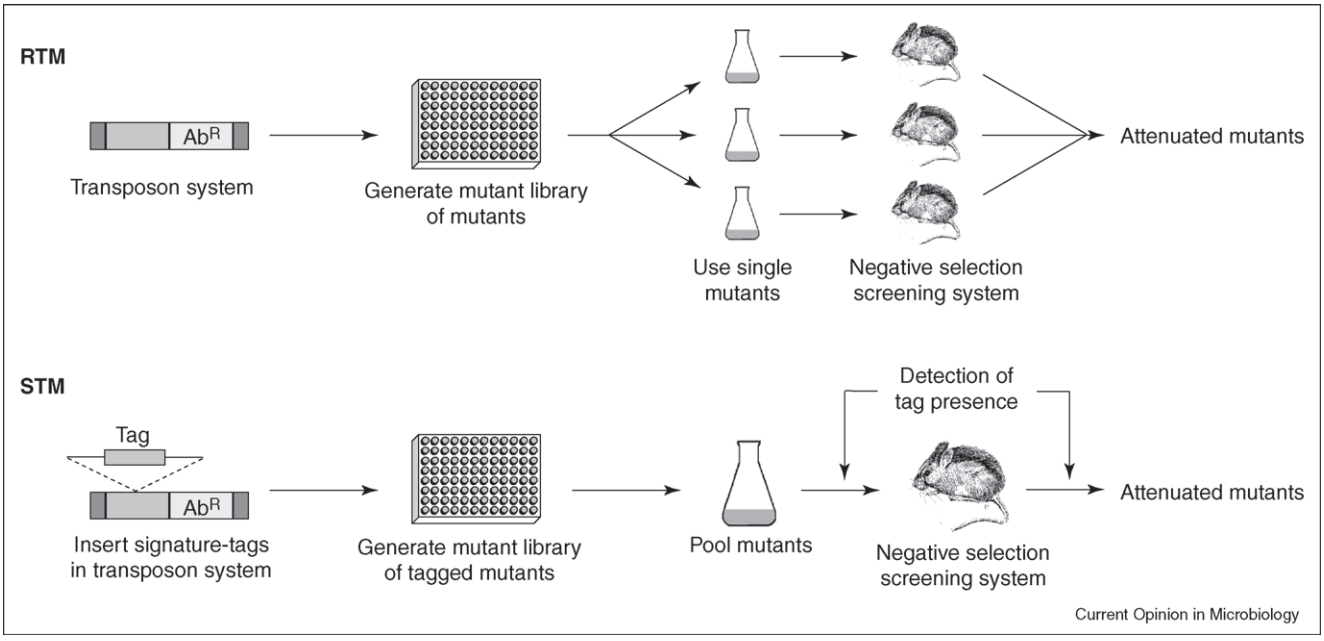
The availability of complete genome sequences for most bacterial pathogens increased substantially the number of genes with unknown function. Genome-wide approaches to functionally characterize these genes in the process of infection have become of great importance. Gene-disruption strategies, such as random transposon mutagenesis, produce insertion mutants that can be tested for attenuated virulence (e.g. in an animal infection model). The isolation of attenuated mutants thus leads to the identification of genes or operons that are required for survival in the infected host. Before the invention of signature-tagged mutagenesis (STM) ten years ago by David Holden and co-workers [1], these mutants had to be screened one by one; however, STM combines the power of insertional mutagenesis and negative selection with a detection system, which allows one to identify individual attenuated mutants from a complex mutant pool (Figure 1). To this end, STM uses signature-tags (i.e. short individual DNA sequences) inserted in the transposons to mark mutants individually. Mutants that carry distinct signature-tags are pooled and injected into the animal host to test in parallel for their survival. This is advantageous as it minimizes both the work-load and the number of animals required.

Owing to its frequent application, STM has been reviewed extensively in recent years [2–7]. Review articles have compared technical variations in STM studies [5], have presented limitations of the STM approach [2,3], and have summarized the results of STM studies until 2000 [4] and 2001 [6]. In this review, we highlight the modular structure of this powerful negative selection method and focus on the technical advances since 2002.

Modular structure of the signature-tagged mutagenesis approach

For the purpose of this review, we present STM as a flowchart of interconnected modules, as depicted in Figure 2. The original STM approach described by Hensel *et al.* [1] was designed to detect new virulence genes of the target organism *Salmonella typhimurium* in a murine model of typhoid fever. To this end, miniTn5 transposons that contained signature-tags composed of random sequences of 40 bp were randomly inserted into the bacterial genome, yielding a tagged mutant library. To validate the suitability of individual tags for detection within a pool of differently tagged mutants, single mutants were pooled and used for test hybridizations. Therefore, the tags of a pool were polymerase chain reaction (PCR)-amplified with universal tag primers, radioactively labeled, and hybridized on membranes spotted with DNA from the corresponding mutants. Only mutants with clear tag hybridization signals were included in the subsequent selection process. Input pools of 96 mutants were subjected to a negative selection system, in this case a mouse infection model. The corresponding output pools recovered after selection were grown on complex medium and their tags amplified and labeled for detection. A weak or absent hybridization signal from the output pool compared to the input pool identified attenuated mutants (Figure 3a). These mutants were tested by different means (e.g. for competition with wild-type bacteria in mixed infections) to validate the screening results. Identification of the mutation site by cloning and sequencing revealed known virulence genes, but also genes previously unrelated to virulence and those with unknown function. Most strikingly, further characterization of selected mutants led to the discovery of a novel *Salmonella* pathogenicity island (SPI-2) [8].

Figure 1



Comparison of standard random transposon mutagenesis (RTM) and signature-tagged mutagenesis (STM), displaying similarities and differences between these two methods.

Since this initial STM study, numerous STM screens have followed a similar protocol. Modifications within individual modules have increased the versatility of the STM method. Some target organisms, such as *Neisseria meningitidis*, are refractory to transposon mutagenesis, leading Sun *et al*. [9] to use *in vitro* mutagenesis and homologous recombination to assemble the tagged mutant library. Other STM studies have used two different negative selection systems [10] or have re-screened to validate their initial screen results by constructing new pools with attenuated mutants and submitting them to a second screen under the same or similar conditions as in the initial screen [11]. In addition, profound changes have been made to some of the modules of the STM screen. For tag validation, Mei *et al*. [12] introduced pre-selection of tags that showed reproducible detection and no cross-reactivity. Each tagged transposon could be subsequently used separately to generate a large amount of tagged mutants. Many STM studies adopted this procedure or directly used the pre-selected tags from previous studies, facilitating the establishment of the method for the specific needs of the study. Also, the way in which mutants are detected has changed profoundly from the original STM methodology. Lehoux *et al*. [13] introduced PCR detection instead of hybridization (Figure 3b).

Recent advances in signature-tagged mutagenesis

In recent years, many new STM studies have been carried out (Table 1). We summarize the major technical changes in the different modules of these STM studies (Table 2).

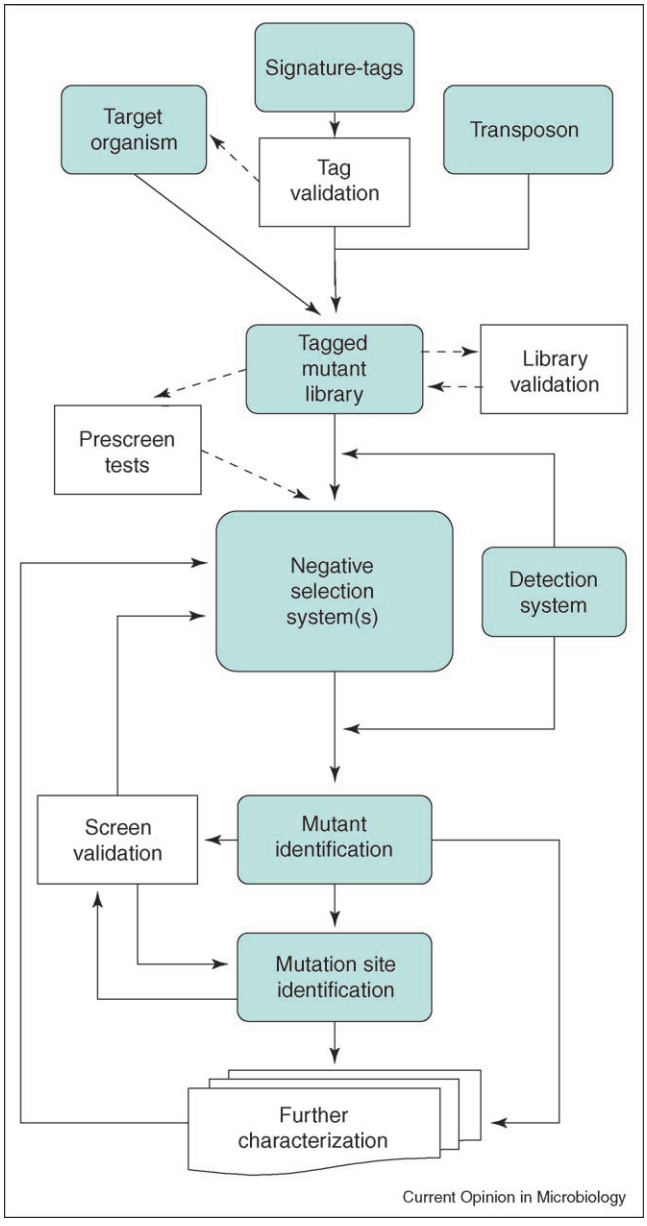
Target organism

Although most STM studies examine pathogen–host interactions, the method is not limited to this application. One recent study investigated the symbiont–host interaction for *Xenorhabdus nematophila* in its nematode host *Steinernema carpocapsae* [14] and another studied the commensal–host interaction for *Campylobacter jejuni* in chicken [15•].

Transposon

Most studies to date have applied the miniTn*5* transposon system [16], which was used in the original STM screen [1] for tag-delivery and mutation of the chosen target organism. This system works in γ-Proteobacteria, among others, but as host factors are required for transposition and owing to target DNA composition, some bacteria are (nearly) refractory to random mutagenesis by Tn*5*-derived transposons. For this reason, several recent studies in *Streptococcus pneumoniae*, *N. meningitidis* and *C. jejuni* [15•,17,18] applied transposons from the *mariner* family, such as *magellan2* or *Himar1* [19]. The activity of these transposons is not dependent on host factors and thus they are applicable to a broad variety of organisms, and only the respective transposase is needed for *in vitro* transposition [20]. The high frequency of transposition and the low insertion-site specificity render these transposons ideal for random transposon mutagenesis [21].

Figure 2



Flowchart overview of the different modules and their interconnectivity in an STM screen. The core components of the STM method are shown in grey/green boxes and the optional components are presented in white boxes. The modules are discussed in more detail in the main text.

Tags

The original STM approach applied signature tags with 40 bp random sequence for hybridization-detection. To allow hybridization-detection on a high-density oligonucleotide array chip, Karlyshev *et al.* [22] used double-tags that had two variable regions of 20 bp (see module 'detection system'). Lehoux *et al.* [13] pioneered detection by PCR, presenting a totally new tag design in their STM study of *Pseudomonas aeruginosa* lung infection [23]. The tags used contained 13 bp of invariant region and a stretch of 7 bp that is variable. By use of tag-specific PCR primers together with a flanking generic primer, every tagged mutant can be detected with a specific PCR reaction.

The common features of tags that are optimized for hybridization or PCR are their constant size and their variable sequence, which are required to discriminate different tags. In size-marker tags, the variable size enables tag discrimination, whereas their sequence is not relevant. Walsh and Cepko [24] first used size-marker tags, but in a totally different context. Two studies on group A *Streptococcus* and *Staphylococcus aureus* adapted this tag design for STM [25,26•]. To construct size-marker tags, Benton *et al.* [26•] cloned 100–600 bp fragments of unrelated DNA in a mutagenesis vector. As these studies used different tag design and an alternative mode of detection, they called their techniques polymorphic-tag-lengths-transposon-mutagenesis [25] and size-marker identification technology [26•]. Nevertheless, these techniques represent variations of STM.

Tagged mutant library

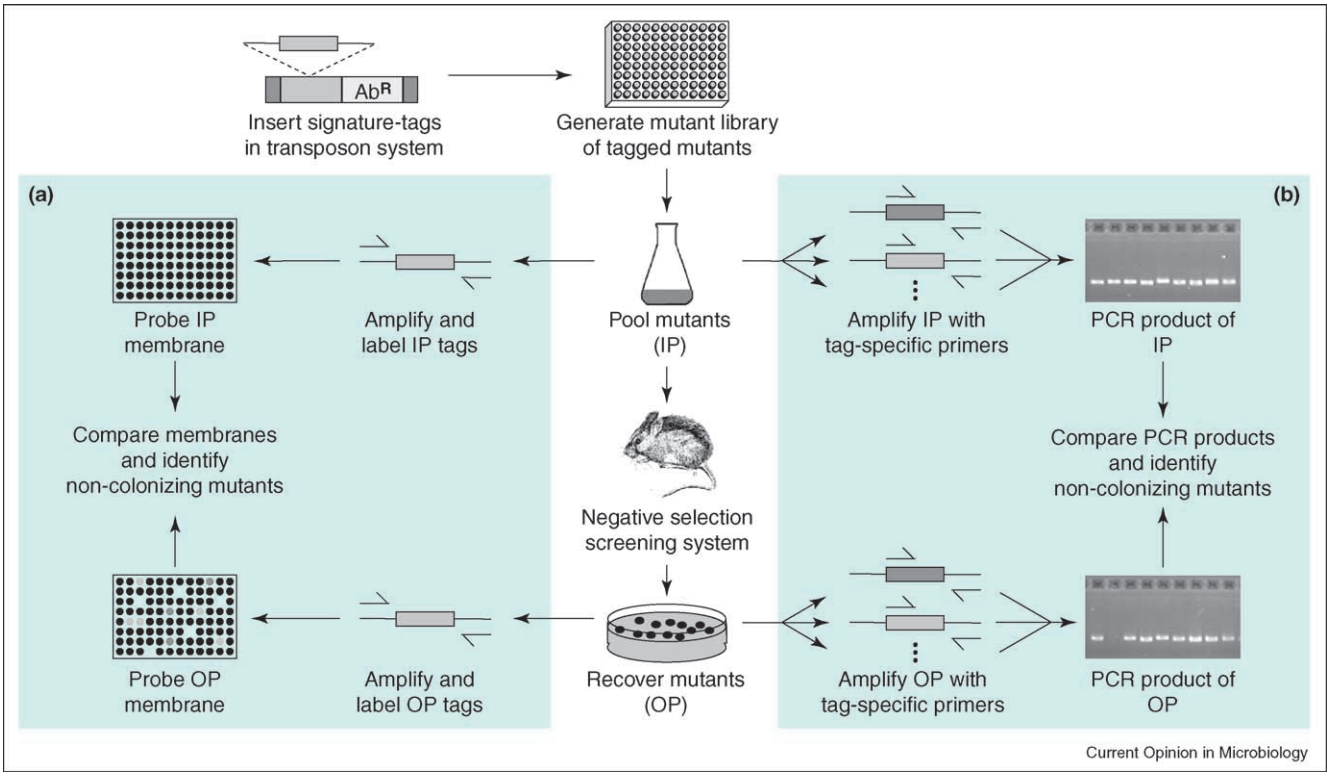
Benton *et al.* [26•] employed pre-tagging of the target organism before mutagenesis. Therefore, they integrated the size-marker tags by site-directed mutagenesis in the *S. aureus* genome without impairing virulence [26•]. Thus, the random mutagenesis is independent from the tagging step. The tag-insertion site must be chosen carefully and even extensive testing does not exclude a changed *in vivo* behavior of the target organism.

In all STM studies, a library of tagged mutants is assembled. During this assembly, bacteria are cultivated on plates or in liquid medium. Therefore, mutants with transposon insertions in essential genes for growth are excluded. By choosing defined culture conditions, specific mutants can additionally be excluded, for example auxotrophic mutants by the use of minimal medium [27,28]. Not associated with virulence per se, specific auxotrophies indicate the nutritional abundance or limitation inside different host niches. However, this exclusion procedure enables exact tailoring of the desired mutant library.

Negative selection system(s)

The negative selection system is the central module in the STM technique. *In vivo* systems have a high selection pressure and are, therefore, the screening system of choice. This is reflected by their broad application in STM studies. Additionally, the use of genetically modified (e.g. knockout or transgenic) animals extends the versatility, as shown in the study of the counter-immune strategy of *Mycobacterium tuberculosis* in immunodeficient mice [29]. In the absence of an adequate *in vivo* system, *in vitro* cell culture systems can represent an alternative. However, *in vitro* results do not necessarily mirror *in vivo* behavior; for example, a comparison of *Klebsiella pneumoniae* mutants obtained by *in vivo* (mice) and *in vitro*

Figure 3



Comparison of two signature-tag detection methods in STM. **(a)** The original STM method, as developed by Hensel *et al*. [1], in which mutants were detected by hybridization. From the mutant library, duplicate hybridization membranes are prepared (colony blots, dot blots with PCR product or dot blots with plasmid DNA). Differently tagged mutants are pooled and an aliquot is used for the preparation of the input pool (IP) hybridization probe. The mutant pool is subjected to the negative selection screening system (e.g. an animal model of infection) and mutants that survive this screen are recovered for preparation of the output pool (OP) hybridization probe. PCR amplification and labeling yields the input and output pool probes used to hybridize on the previously prepared membranes. The output and the input pool membranes are then compared. Mutants that fail to be recovered from the negative selection screen produce a signal on the input pool membrane, but not on the output pool membrane. **(b)** STM with PCR detection, as developed by Lehoux *et al*. [13]. After pooling of differently tagged mutants from the mutant library, an aliquot from the input pool is used for detection of the individual tags by amplification with tag-specific primers. For each tag, one PCR reaction is prepared. The mutant pool is subjected to the negative selection screening system and mutants that survive this screen are recovered for detection of the individual tags of the output pool by amplification with tag-specific primers. Mutants that fail to be recovered from the negative selection screen are identified as producing a PCR product with the input pool as template, but not with the output pool as template.

(intestine cells) selection revealed a minimal overlap of only one gene required in both conditions [30]. STM can also be applied in the absence of an animal or cell culture model as the only requirement is a negative selection system. Geoffroy *et al*. [18] tested *N. meningitidis* for factors required for serum resistance in a cell-free system.

Detection system

As outlined previously (Figure 3), hybridization and PCR are two different detection methods. A special type of detection by hybridization is the high-density oligonucleotide array technique, in which the presence or absence of 192 individual tag sequences that correspond to 96 double-tags can be detected separately [22]. When an STM study includes several negative selection screens, the multi-screening approach of Struve *et al*. [31] reduces work-load. This study describes a reversion of a dot blot hybridization protocol by spotting the amplified tags from the output pool on a membrane and then probing this with an amplified and labeled probe that contains all tags used for pool generation. The advantage of this change is that the number of hybridizations necessary to screen the mutant library in more than one screening system is substantially reduced because pools of tags recovered from more than one screening system can be analyzed simultaneously [31].

Many recent STM studies have used PCR detection, which was established by Lehoux *et al*. [13] and then applied in their recent *P. aeruginosa*-STM study [23]. The basic principles of this method are depicted in Figure 3b. The tags are amplified individually with a tag-specific primer and a generic primer in a constant region flanking the tag. The presence of a PCR product of one tag in the input pool and absence of a PCR product for the same tag in the output pool shows the loss of the specific tagged

Table 1

STM studies in bacteria (2002 to present).

Species[a]	Route of infection[b]	Host	Niche[c]	Mutants (attenuated versus screened)		Pool size	Reference
				Numbers	%		
S. e. Typhimurium	ip	Mice	Spleen	40/1152	3.5	96	[1]
S. e. Typhimurium	Oral	Calves, chicken	GI tract	84/1045	8.0	95	[35]
S. e. Choleraesuis	Oral, ip	Swine	GI tract, spleen	3/45	6.7	45	[36]
Escherichia coli (UPEC)	tu	Mice	Urinary tract	19/2049	0.9	46	[37]
E. coli (EHEC)	Oral	Calves	GI tract	79/1900	4.2	95	[27]
E. coli (EHEC)	Oral	Calves	GI tract	62/570	10.9	95	[38]
E. coli (APEC)	it	Chicken	Spleen	30/1800	1.7	90	[39]
Vibrio cholerae	Oral	Mice	Small intestine	251/9600	2.6	96	[33]
Yersinia pestis	sc	Mice	Spleen	16/300	5.3	20	[34•]
Citrobacter rodentium	Oral	Mice	Colon	14/576	2.4	24	[40]
Klebsiella pneumoniae	Oral	Mice, cell culture	Colon, intestinal cells	29/2200	1.3	48	[30]
K. pneumoniae	Oral, tu	Mice	Colon, bladder	19/1440	1.3	48	[31]
Proteus mirabilis	tu	Mice	Bladder	32/2088	1.5	47	[41]
Xenorhabdus nematophila	Contact	Nematode	Intestinal vesicle	15/3000	0.5	48	[14]
Pasteurella multocida	ip, im	Mice, chicken	Blood	15/420	3.6	42	[42]
Actinobacillus pleuropneumoniae	it	Swine	Lung	105/2064	5.1	48	[43]
Haemophilus influenzae	ip	Rats	Blood	24/1632	1.5	24	[44]
Pseudomonas aeruginosa	it	Rats	Lung	13/1056	1.2	11	[23]
P. aeruginosa	it	Rats	Lung	160/7968	2.0	72	[32]
Brucella melitensis	ip	Mice	Spleen	36/1152	3.1	96	[45]
Neisseria meningitides			Serum resistance	37/4548	0.8	44-48	[18]
Burkholderia pseudomallei	ip	Mice	Spleen	1/96	1.0	96	[46]
Burkholderia cenocepacia	it	Rats	Lung	102/2627	3.9	37	[28]
Campylobacter jejuni	Oral	Chicken	Cecum	29/1550	1.9	74-82	[15•]
Helicobacter pylori	Oral	Mongolian gerbils	Stomach	252/960	26.3	24	[47]
Staphylococcus aureus	ip	Mice	Spleen	24/6300	0.4	50	[26•]
Streptococcus pneumoniae	in	Mice	Lung	387/6149	6.3	63/40	[17]
Streptococcus group A (GAS)	sc	Mice	Spleen	1/21	4.8	21	[25]
Mycobacterium tuberculosis	iv	Mice (knockout)	Lung, spleen, liver	3/48	6.3	48	[29]
Mycobacterium marinum	ip	Goldfish	Liver	40/1008	4.0	48-53	[48]
Mycobacterium bovis	sc	Guinea pigs	Spleen	15/1215	1.2	45	[49]

[a] APEC, avian pathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; *S. e.*, *Salmonella enterica*; UPEC, uropathogenic *E. coli*.
[b] im, intramuscular; in, intranasal; ip, intraperitoneal; it, intratracheal; iv, intravenous; sc, subcutaneous; tu, transurethral.
[c] GI, gastrointestinal.

mutant during the negative selection screen. An elegant way in which to screen 72 mutants simultaneously with a tag-pool size of 24 Lehoux-tags was shown by Potvin *et al.* [32]. They used three transposons that differed only in the incorporated selection markers. For detection, this study employed multiplex PCR, using three selection marker-specific primers instead of one generic primer. However, unlike hybridization, PCR detection does not allow different attenuation levels to be distinguished. This limitation led Hunt *et al.* [28] to introduce real-time PCR detection. Real-time PCR allows the relative quantification of template DNA, in this case of the different mutants represented in a pool [28].

Mutation site identification

To date, one of the major shortcomings of STM has been the inability to detect whether a certain gene is dispensable during negative selection or whether it is simply not present in the mutant library. Only one approach, published by Geoffroy *et al.* [18], aimed to map the transposon insertion sites of all mutants tested in their STM screen to answer this question. Despite the knowledge of mutational coverage, this comprehensive mapping is not a preferential strategy owing to the high time and labor consumption.

Further characterization

The STM method can be reused for testing randomly chosen or deliberately selected mutants in pools. Merrell *et al.* [33] subjected a pool of selected *Vibrio cholerae* intestinal colonization-attenuated mutants, denominated virulence-attenuated pool (VAP), to a cell-free assay for acid shock. In addition, further analyses can comprise testing of mutants individually for their specific phenotype. Thereby, STM has the advantage that the output of the selection are interesting mutant strains, which can be used directly for further investigation, for example to evaluate possible vaccination targets in the human pathogen *Yersinia pestis* [34•].

Table 2

Technical details of the STM studies presented in Table 1.

Species[a]	Transposon	Tags	Detection system[b]	Mutation site identification[c]	Special features[d]	Reference
S. e. Typhimurium	miniTn*5*	Hensel-tags	rh (cb)	c, s	First STM screen	[1]
S. e. Typhimurium	miniTn*5*	Hensel-tags	rh (dbp)	c, s	Two negative selection systems	[35]
S. e. Choleraesuis	miniTn*5*	Hensel-tags	rh (cb)	c, s		[36]
Escherichia coli (UPEC)	miniTn*5*	Hensel-tags	nrh (dbpl)	Arbitrary PCR / c, s		[37]
E. coli (EHEC)	miniTn*5*	Hensel-tags	rh (cb)	c, s	Auxotrophic exclusion	[27]
E. coli (EHEC)	miniTn*5*	Hensel-tags	rh (cb)	c, s		[38]
E. coli (APEC)	miniTn*5*	Hensel-tags	nrh (dbp)	Arbitrary PCR, s		[39]
Vibrio cholerae	miniTn*5*	Hensel-tags	nrh (dbpl)	c, s	Virulence-attenuated pools	[33]
Yersinia pestis	miniTn*5*	Hensel-tags	rh (dbpl)	Single primer PCR, s	Vaccine candidate testing	[34•]
Citrobacter rodentium	miniTn*5*	Hensel-tags	rh (dbpl)	c, s		[40]
Klebsiella pneumoniae	miniTn*5*	Hensel-tags	rh (cb)	c, s	Two negative selection systems	[30]
K. pneumoniae	miniTn*5*	Hensel-tags	nrh (dbp)	c, s	Multi-screening STM	[31]
Proteus mirabilis	miniTn*5*	Hensel-tags	nrh (dbpl)	Arbitrary PCR / c, s		[41]
Xenorhabdus nematophila	miniTn*5*	Hensel-tags	rh (dbpl)	c, s	First symbiosis STM	[14]
Pasteurella multocida	Tn*916*	Hensel-tags	nrh (dbp)	Inverse PCR, s		[42]
Actinobacillus pleuropneumoniae	mini Tn*10*	Hensel-tags	rh (cb)	c, s	Vaccine candidate testing	[43]
Haemophilus influenzae	Tn*1545*	Hensel-tags	rh (cb)	Arbitrary PCR, s		[44]
Pseudomonas aeruginosa	miniTn*5*	Lehoux-tags	PCR	c, s	First application of PCR detection	[23]
P. aeruginosa	miniTn*5*	Lehoux-tags	PCR (multiplex)	c, s	Multiplex PCR detection	[32]
Brucella melitensis	miniTn*5*	Hensel-tags	rh (dbp)	Arbitrary PCR / inverse PCR, s	Sequel to [50]	[45]
Neisseria meningitidis	*Himar1*	Hensel-tags	rh (cb)	Ligation-mediated PCR, s	First cell-free screen	[18]
Burkholderia pseudomallei	miniTn*5*	Hensel-tags	rh (cb)	c, s		[46]
Burkholderia cenocepacia	pTnMod	Lehoux-tags	PCR (real-time)	Self-cloning, s	Real-time PCR detection, auxotrophic exclusion	[28]
Campylobacter jejuni	*Himar1* derivative	Hensel-tags	nrh (dbp)	c, s	First commensal STM	[15•]
Helicobacter pylori	Tn*Max5*	Hensel-like tags (20 bp)	PCR	c, s		[47]
Staphylococcus aureus	Tn*551* and Tn*917lac*	Size-marker tags	PCR (real-time)	Inverse PCR, s	SMIT, pre-tagging	[26•]
Streptococcus pneumoniae	*magellan2*	Hensel-tags	nrh (dbp)	Arbitrary PCR, s	Two-stage STM	[17]
Streptococcus group A (GAS)	IS*256*	Size-marker tags	PCR	Self-cloning, s	PTTM	[25]
Mycobacterium tuberculosis	Tn*5370*	Hensel-tags	rh (cb)	Inverse PCR, s	Knockout mice as model	[29]
Mycobacterium marinum	phasmid	Hensel-tags	rh (dbpl)	c, s		[48]
Mycobacterium bovis	illegitimate recombination	Hensel-tags	rh (sbpl)	c, s		[49]

[a] APEC, avian pathogenic *E. coli*; EHEC, enterohemorrhagic *E. coli*; *S. e.*, *Salmonella enterica*; UPEC, uropathogenic *E. coli*.
[b] cb, colony blot; dbp, dot blot with PCR product; dbpl, dot blot with plasmid DNA; hdh, high-density hybridization on chip; nrh, non-radioactive labeling and hybridization; rh, radioactive labeling and hybridization; sbpl, Southern blot with plasmid DNA.
[c] c, cloning; s, sequencing.
[d] PTTM, polymorphic-tag-length-transposon-mutagenesis; SMIT, size marker identification technology.

Conclusions

One decade after its first description, STM has become a genetic method widely used for the *in vivo* identification of virulence traits in pathogenic bacteria. Recent technical advances, mainly in the choice and combination of negative selection system(s) and in the choice of detection systems, have broadened its applicability and versatility. The STM method is an invaluable tool to provide a better understanding of microbial behavior *in vivo*. The use of other genome-scale techniques such as *in vivo* expression technology, microarray analysis, genome analysis and mapping by *in vitro* transposon mutagenesis, or transposon site hybridization complement the lessons learned by STM.

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Manuscript in preparation

Functional genomics of *Bartonella* pathogenesis: Large-scale signature-tagged mutagenesis reveals a high number of genes required for infection of the mammalian host

Saenz H. L.[1], Stoeckli M. C.[1], Vayssier-Taussat M.[2], Lanz C.[3], Schuster S. C.[3], Dehio C.[1,*]

[1]*Division of Molecular Microbiology, Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland,*
[2]*ENVA, UMR 956, Microbiologie-Immunologie, 94 700 Maisons-Alfort, France,*
[3]*Max-Planck-Institute for Developmental Biology, 72076 Tuebingen, Germany.*

Running title: STM screen for pathogenicity factors in *Bartonella*

*Corresponding author: Prof. Christoph Dehio
Division of Molecular Microbiology
Biozentrum, University of Basel
Klingelberstrasse 70
CH-4056 Basel, Switzerland
Tel. +41-61-267-2140
Fax: +41-61-267-2118
E-mail: christoph.dehio@unibas.ch

Author contributions: H.L.S., C.D. designed research; H.L.S., M.C.S., M.V., C.L. performed research; S.C.S. contributed analytic tools; H.L.S., C.D. analyzed data; and H.L.S. wrote the paper

ABSTRACT

Bartonellae are bacterial pathogens uniquely adapted to cause intraerythrocytic infection in their mammalian reservoir hosts. In the case of human-specific *Bartonella bacilliformis* and *Bartonella quintana*, the intraerythrocytic bacteremia leads to the clinical manifestations of Oroya fever and trench fever, respectively. Here, we adapted large-scale signature-tagged mutagenesis (STM) for the first time to *Bartonella,* allowing us to screen for pathogenicity factors required for infection of the mammalian reservoir host *in vivo*. A total of 3084 STM mutants of rat-specific *B. tribocorum* were screened in a rat infection model for these criteria. After two rounds of screening, 130 mutants showed severe attenuation compared to wild-type *B. tribocorum*. We mapped the transposon insertion sites of these mutants to 80 different genes, and categorized them according to their putative function. Besides already described pathogenicity factors responsible for interaction with the host, like the two type IV secretion systems VirB-D4 and Trw, we discovered factors previously unlinked to pathogenesis. These belong to diverse functional classes, like transport, gene-expression regulation, cell envelope integrity, or metabolism. A quarter of the identified genes are (conserved) hypothetical coding for novel pathogenicity factors. We have used an additional PCR-screening approach on the entire mutant library to test for the level of mutational saturation and to identify non-essential genes in a pathogenicity island encoding 18 gene products related to the process of type IV secretion.

INTRODUCTION

Bartonellae are small, fastidious, pleomorphic, Gram-negative rods, which are pathogenic for a wide range of mammalian hosts. The genus *Bartonella* currently comprises 20 species that are highly adapted to their mammalian reservoir hosts. Of these 20 species, 8 have been associated with human disease (20). The three major human pathogens are the human-specific *Bartonella bacilliformis* and *Bartonella quintana* and the cat-specific *Bartonella henselae*, where humans appear as incidental hosts. The common theme of *Bartonella* infections in the reservoir host is the long-lasting intraerythrocytic bacteremia. The course of infection is most frequently asymptomatic, but can also lead to severe clinical manifestations like Oroya fever in the case of *B. bacilliformis* or trench fever in the case of *B. quintana*. Both in the incidental and the reservoir host, *Bartonella* interacts also with endothelial cells which in a immunocompromized individual can cause vasoproliferative lesions like verruga peruana in the case of *B. bacilliformis* or bacillary angiomatosis in the case of *B. quintana* and *B. henselae* (10).

Recently, Schulein et al. developed an animal model for erythrocyte colonization of *Bartonella* in the reservoir host (73). After injection of rat-specific *Bartonella tribocorum* in the tail vein of rats, bacteria are rapidly cleared from the circulating blood. Plate-grown bartonellae obviously are not able to directly invade erythrocytes and are not detected in the blood on the first days of infection. Thus, they first have to interact with a yet not experimentally identified primary niche, where upon invasion they become competent for the subsequent hemotropic stage (73), a process including transcriptional reprogramming. So, colonization of erythrocytes and persistence therein is the endpoint of a complex series of bacterium-host interactions. To understand such a complex pathogenesis including interaction with and invasion into different host cell types, identification of involved pathogenicity factors of the bacteria are of prime significance. Here, pathogenicity is defined in a broad sense, i.e. the ability of a microorganism to breach barriers in the host and to thrive either hidden from or in the face of the host immune defense. Intraerythrocytic bacteremia is the hallmark of *Bartonella* infection in the reservoir host and is responsible for the disease symptoms, elicited by the human-pathogenic bartonellae mentioned above. So, bacteremia can be used as a read-out, whether mutant bacteria are still able to colonize the intraerythocytic niche. Thus, abacteremic mutants then by definition carry a mutation in a gene coning for a pathogenicity factor. Our current knowledge about these pathogenicity factors of *Bartonella* is summarized in the following paragraph. Using the *B. tribocorum*-rat

infection model, Schulein and Dehio (71) described mutants in components of the VirB-D4 type IV secretion system (T4SS) to be abacteremic, proving these components to be the first *bona fide* pathogenicity factors in *Bartonella*. Seubert et al. discovered a second T4SS, the Trw system, and also could show its essential role as pathogenicity trait in the rat-infection model (74). Very recently, Riess et al. identified a surface-expressed, afimbrial adhesin of *B. henselae* designated as *Bartonella* adhesin A (BadA), formerly known as "type IV pilus" (3) to be a major pathogenicity factor (61). A family of variably expressed outer-membrane proteins (VompA-D) in *B. quintana*, orthologous to BadA, supports these findings (91).

Further possible pathogenicity factors of *Bartonella* have been reviewed recently (20) and include (i) a yet not clearly characterized proteinaceous angiogenic factor, found in *B. bacilliformis, B. quintana,* and *B. henselae*, (ii) a secreted bacterial factor called deformin of *B. bacilliformis* and *B. henselae*, involved in the deformation of erythrocyte membranes, (iii) the flagella of *B. bacilliformis,* facilitating erythrocyte invasion, (iv) unusual lipopolysaccharide with low endotoxic activity of *B. henselae* and *B. quintana*, enabling the interaction with endotoxin-sensitive endothelial cells, (v) hemin-binding proteins (HbpA-E of *B. quintana* and HbpA-D of *B. henselae*), involved in iron acquisition, (vi) the surface-exposed proteinaceous hemolysin of *B. bacilliformis*, responsible for hemolysis during Oroya fever, (vii) the inducible *Bartonella* autotransporter (Iba) family, specifically upregulated during endothelial infection by *B. henselae in vitro* and transiently activated during rat infection by *B. tribocorum in vivo*, (viii) the two gene products IalA and IalB of the invasion-associated locus (*ial*) mediating erythrocyte invasion by *B. bacilliformis in vitro*, and (ix) multiple outer membrane proteins of *B. henselae* demonstrated to bind to endothelial cells *in vitro* (20).

The sequencing of two *Bartonella* genomes, namely of *B. quintana* and *B. henselae,* published by Alsmark et al. in 2004 (1), opened the way for whole-genome approaches to comprehensively study pathogenicity factors in *Bartonella*. Cellular models can be applied to study aspects of the versatile bacterium-host interactions, but *in vivo* studies better depict the complex natural situation. A powerful technology for *in vivo* screening is signature-tagged mutagenesis (STM) (30), which allows simultaneous screening of pools of transposon mutants for loss of pathogenicity in an animal-infection model. Thus, factors identified by STM are essential for colonization in the tested model, a unique feature of STM compared to other *in vivo* screening techniques. The application of this technique identified many new pathogenicity factors in a broad spectrum of pathogenic bacteria and fungi (recently reviewed by Mecsas [49]). In STM, the transposon insertion mutants are individually marked with a

specific signature-tag, i.e. a short variable double-stranded DNA sequence. In pools of mutants, the presence or absence of a single mutant can be tested by detection of this tag. Originally, Hensel et al. used DNA hybridization as detection method (30), but more recently, Lehoux et al. described mutant detection by PCR as a simpler and more rapid detection technique (43), which could be even automated for high-throughput screening (42). Pools of mutants can be screened in an animal model, cell culture, or any other negative selection screen. The presence of an individual mutant in the pool before selection and its absence thereafter identifies the mutant as a non-colonizing mutant candidate. Since STM uses mutant pools, where different mutants influence each other, some STM screens employed a rescreening of the non-colonizing candidates in newly assembled pools to discriminate between fully attenuated mutants, which are attenuated in every pool tested, and partially attenuated mutants, which are present or not depending on the pool composition. Darwin and Miller (18) first described this two-stage STM.

Here, we established large-scale STM for *Bartonella* to identify pathogenicity factors of *B. tribocorum* essential for inducing intraerythrocytic bacteremia during infection of the laboratory rat as the mammalian reservoir host. By screening 3084 transpositional mutants for an abacteremic phenotype, we discovered novel pathogenicity factor as well as factors previously unlinked to pathogenesis and re-discovered known pathogenicity factors, like the two T4SS VirB-D4 and Trw. We have used an additional PCR-screening approach on the entire mutant library to test for the level of mutational saturation and to identify non-essential genes in a pathogenicity island encoding 18 gene products related to the process of type IV secretion.

RESULTS

Construction and testing of the transposon vector

The transposon vector pHS003 contains an oriT for conjugative transfer, the *Himar1* transposon, carrying a kanamycin resistance marker, and a hyperactive transposase (Figure 1). This suicide vector construct was transferred into *B. tribocorum* via conjugation and transconjugants were tested for transposon insertions, leading to kanamycin-resistant colonies. The frequency of transposition events (transconjugants divided by *B. tribocorum* recipients) was $2.4x10^{-4}$. With specific PCR reactions on single colonies, we could amplify a part of the transposon, but no PCR product could be obtained with primer pairs derived from the vector backbone, indicating insertion of the transposon into the genome of *B. tribocorum* and contradicting a potential integration of the whole plasmid (data not shown). Southern analysis (Figure 2) confirmed this indication and showed that in each of the 13 randomly chosen mutants, the transposon inserted in a single copy in distinct sites of the chromosome, suggesting random transposon distribution.

Construction of the STM mutant library

A mixture of signature-tags was produced by PCR using degenerate oligonucleotide templates, ligated into transposon vector pHS006 (derivative of pHS003, see "Material and Methods"), and transformed into *Escherichia* coli NovaBlue. The central variable sequence of the tag allows the potential generation of more than 10^{22} different variants. Ninety-six ampicillin/kanamycin-resistant colonies were picked (*E. coli* NovaBlue pHS006tag*n* with *n* = 001-096) and the inserted tag was sequenced. None of the plasmids contained identical tag sequences (data not shown). We chose, based on reproducible PCR detection results, 36 plasmids to construct the STM mutant library, and transferred the tagged transposon vectors individually to *B. tribocorum* via conjugation. The overall frequency of transposition events of 36 individual conjugation assays presented as $2.3x10^{-4}$. From each conjugation assay, we selected 96 single kanamycin-resistant colonies. Using this procedure, we assembled an STM mutant library with 3456 mutants.

Screening of the mutant library

The first 16 mutant pools consisted of 19 differently tagged mutants, the remaining 80 mutant pools of 36 differently tagged mutants. Each mutant pool was used to inoculate two rats (= input pool, see also "Material and Methods") and peripheral blood was drawn on days 7 and

14 postinfection. Bacteria were recovered from the blood by freeze-lysation and grown on plates (=output pool). To identify abacteremic mutant candidates, present in the input pool and missing in the output pool of both rats on both time points investigated, we compared the individual tag-specific PCR signals in input and output pool. Of 3184 mutants tested, 100 had to be excluded due to missing input pool signal. One tag (tag047) was excluded from all pools due to constantly weak PCR detection results. Of the remaining 3084 mutants tested, 359 were abacteremic mutant candidates. To confirm this abacteremic phenotype, we reassembled the 359 strains into 18 pools of 34 mutants. For keeping the pool size constant at 34 mutants per pool, we tested some strains repeatedly. In this rescreen, the pools were used to inoculate four rats, two like in the primary screen and two competitively mixed with a pool of 5 mutants that showed wild-type behavior in the primary screen and all contained the same tag. This pool was called the tagged wild-type-like pool. Since the mutants in this pool showed wild-type behavior in the screen and carried mainly intergenic transposon insertions (data not shown), we assumed that this pool should be competitive like wild-type bacteria. The rescreen revealed 130 abacteremic mutants of the 359 candidates, which corresponds to 4% of the 3084 mutants tested. In total, these 130 abacteremic mutants were tested absent from the blood of at least 6 rats (Table 1) on two time points, where bacteremia peaks with wild-type *B. tribocorum*. All abacteremic mutants have a calculated in-pool competitive index (CI, see "Material and Methods") lower than 0.00001. The *in vitro* growth in all abacteremic mutants was comparable to wild-type bacteria.

Interestingly, 47 strains could not be detected from the competition pool, where more wild-type like bacteria were present, but they could be recovered from the mutant pools. This indicates that wild-type like strains outcompeted these otherwise bacteremic strains, which will be called outcompeted mutants in this work.

Mapping of the transposon insertion site

We determined the insertion site of the transposon for 314 of the 359 abacteremic mutant candidates by direct sequencing out of the transposon into the genome of *B. tribocorum*. The remaining 45 mutants gave ambiguous sequencing results, suggesting that these mutants might carry multiple transposon insertions. This finding is supported by Southern analysis, where 20 selected mutants showed unique single bands, whereas two suspected double insertion mutants showed also two bands (data not shown). The 130 abacteremic mutants represent transposition events into 80 different open reading frames (ORFs). With the help of the (not yet fully assembled) genome sequence of *B. tribocorum* (of approx. 2.7 Mb)

(S. C. Schuster, P. Engel, H. L. Saenz, M. C. Stoeckli, C. Lanz, G. Raddatz, and C. Dehio, unpublished results) and the two published *Bartonella* genomes of *B. quintana* (1.6 Mb) and *B. henselae* (1.9 Mb) (1), we annotated the affected 80 ORFs, whose predicted products fall into a wide variety of functional classes (Table 1). Remarkably, various multiple hits were found within the *trw* and *virB* gene clusters, coding for the two T4SS known to be essential for rat infection (71, 74).

Categorization of abacteremic mutants

Of the 130 abacteremic mutants, displayed in Table 1, 24 strains carried transposon insertions in intergenic regions. This localization could affect transcriptional termination of the upstream gene, or more likely, disrupt the promoter or regulator region of the downstream gene, or disrupt an operon. Additionally, the transposon could have hit a small not yet discovered ORF, a sequence encoding tRNA or a small regulatory RNA. We included these intergenic mutants in the classification as affecting the downstream gene, if the insertion was not more than 500 bp upstream of a gene and nearer to the downstream gene than to the upstream gene and if there was no indication for a functional small ORF, tRNA gene (predicted with tRNAscan-SE), or a sequence coding for a small regulatory RNA. This issue would nevertheless need further experimental exploration. Thirteen (of thirteen analyzed) intergenic hits could be classified by these criteria. Besides the intergenic hits, 106 transposon insertions occurred within 80 ORFs. These insertions can be divided into 84 hits in 64 genes with predicted putative function, 14 hits in 12 conserved hypothetical genes (CH) with no predicted function, 2 hits in 2 phage-specific genes (not present in *B. henselae* or *B. quintana*), and 6 hits in 2 *Bartonella*-specific genes. Figure 3 shows the functional classification of the affected genes. Genes with products involved in adhesion and invasion or transport over the bacterial membranes and gene-expression regulation constituted more than half of the total hit genes (35 and 14 of 80, respectively), highlighting the importance of these processes in pathogenicity. Twelve genes with products involved in the cellular metabolism, like energy metabolism, amino acid or cofactor biosynthesis, and carbon metabolism were affected. The individual mutants are listed in Table 1 with their assigned classification, their putative assigned function, their orthologs in *B. henselae* and *B. quintana* (if present), and the number of animals, in which they were tested.

Insertional Analysis of the *virB/D4/bep* Cluster

Figure 4 shows the genetic architecture of the *virB/D4/bep* cluster. Herein, the *virB/D4* genes encode a T4SS essential in *B. tribocorum in vivo* (73) and in *B. henselae in vitro* for the phenotypes associated with endothelial cell infections (70, 71). Recently, Schulein et al. discovered the cognate effector proteins encoded by the *bep* cluster downstream the T4SS structural genes (72). Interestingly, Figure 4A reveals many transposon insertions in the T4SS structural genes, but only one insertion in one of the effector genes (*bepD1*). This imbalance could reflect absence of further transposon insertions in the *bep* cluster in the entire mutant library or non-essentiality of *bep* genes individually in the *in vivo* situation. To discriminate between these two possibilities, we established a screening method for transposon insertions in a selected gene on the basis of the whole mutant library. This method is based on PCR with a transposon-specific primer and a primer flanking the gene of interest. Using the 96 input pools as template, we identified input pools, in which a PCR product indicated presence of a transposon insertion in the gene of interest. Sequencing of the PCR product determined the corresponding signature-tag and, thus, the mutant and the exact insertion site of the transposon. With this test, we confirmed abacteremic mutants in *virD4, virB10,* and *virB9* and revealed the presence of transposon insertion mutants in *bepA, bepD1, bepD2,* and *bepF* in the library (Figure 4B, Table 2), but no insertions in the tested genes *virB8, virB11, bepB, bepC,* and *bepE*. Table 2 shows that most of the *bep* gene mutants behaved like wild-type bacteria in the primary screen and were, thus, not further investigated in the rescreen. One puzzling result of this analysis is the observation that one mutation in *bepD1* led to the abacteremic phenotype, whereas several other mutations in the same gene conferred a wild-type like bacteremic phenotype.

Individual animal experiments

To prove the difference between the abacteremic and the bacteremic phenotype of the *bep* mutants and to quantify possible attenuation, competition analyses of selected *bep* mutants with wild-type bacteria were done and CI were calculated (Table 2). All the tested *bep* mutants, except the *bepD1* abacteremic mutant, did not show attenuation compared to wild-type bacteria, neither in individual nor in competitive infections. The *bepD1* abacteremic phenotype was confirmed in all animals tested.

DISCUSSION

STM screen results

We tested the ability of transposon insertion mutants of *Bartonella* to cause intraerythrocytic bacteremia, the central colonization niche of *Bartonella* in the reservoir host, to identify pathogenicity factors essential for host-colonization. Our results demonstrate that STM effectively identifies *B. tribocorum* genes essential for pathogenicity in the rat-infection model. Additionally to genes already known to be important for pathogenicity, we have identified a large number of loci previously unlinked to virulence. For interpretation of the results, it is of major importance to keep in mind that a mutation derived by transposon insertion not only disrupts the respective gene, but may cause polar effects on expression of downstream genes in the same operon. If the operon contains functionally related genes, the insertion remains informative. Furthermore, STM is based on the premises that each member of a population of bacteria has an equal opportunity to establish an infection and that each organism acts independently. Reality is more complex as there also will be positive or negative interaction between different mutants in one pool, like extracellular complementation or outcompetition. In the rescreen, outcompetition behavior could be shown by coinfection with wild-type like competition pools, where some mutants were outcompeted, whereas they thrived in mutant pools with less competitional power. All these mutants could be interesting to investigate for a better understanding of bacterial competition factors inside the host. But this study concentrated only on those mutants with a major defect in establishing infection inside the host. Thus, we only included mutants that exhibited the abacteremic phenotype under all conditions tested (screen and rescreen). The affected genes can be functionally classified in five diverse categories: (i) cellular interaction with the host, (ii) gene-expression regulation, (iii) cell envelope integrity, (iv) metabolism, and (v) unknown function. The first category can be further divided into adhesion / invasion and transport processes.

Adhesion and invasion

The mammalian body is an environment usually hostile to invading pathogens, which have to face the immune system and have to compete with host cells for nutrients. The outer membrane is the major device of bacteria to interact with host cells, the extracellular matrix, or soluble factors in body fluids. Therefore, outer membrane proteins can represent important pathogenicity factors for bacterial pathogens and have diverse functions including adhesion, substrate uptake, antimicrobial resistance, and resistance to serum, drugs, or bile. This STM

screen identified many already described pathogenicity factors in *Bartonella* from this category, thereby validating the STM approach.

From these factors, we isolated three mutations in an autotransporter cluster (*ibaB, ibaC, ibaD*). The promoter of *ibaB* was shown to be induced in *B. henselae* during endothelial infection *in vitro* and to be transiently active in *B. tribocorum* at the onset of blood-stage infection *in vivo* (75). Analysis of these autotransporters reveals that they most likely serve as adhesins (Anja Seubert, unpublished results). To our best knowledge, these are the first autotransporters to be reported in an STM study. Autotransporters generally serve as adhesins, extracellularly released degrading enzymes or toxins (recently reviewed by Henderson et al. [29]). Considering their function as secreted enzymes and toxins, extracellular complementation in a pool of mutants could prevent detection of autotransporters in STM screens. As adhesins, at least partial complementation could occur by non-mutated homologous members of the same locus. The screen results show that the function of the autotransporters of the *iba* family must be extracellularly uncomplementable. They may act synergistically as adhesins, e.g. to different structures on erythrocytes during blood stage infection, and thus be all needed because of the adverse conditions for bacteria in the blood stream (e.g. high shearing forces, innate immune responses). Alternatively, they could be differentially expressed in different host tissues. Further investigation is required to determine their exact role in pathogenicity. A further adhesin was affected in mutant 087E05 that carried a transposon insertion directly upstream of *badA*, which codes for an afimbrial adhesin of *Bartonella* related to YadA of enteropathogenic *Yersinia* and NadA of *Neisseria meningitidis*. This adhesin BadA in *B. henselae* mediates binding to extracellular matrix proteins and to endothelial cells *in vitro*, and was additionally shown to be immunodominant in *B. henselae*-infected patients (61). Hypothetical genes that share partial homology with *badA* surround the *badA* gene. The orthologous locus in *B. quintana* comprises the so-called *vomp* genes (*vompA-D*). Their products mediate autoaggregation and also adhesion to extracellular matrix proteins *in vitro*, and the locus exhibits genomic rearrangement during the course of blood stream infection *in vivo* (91). Furthermore, we discovered one transposon insertion inside the *ialB* gene and one directly upstream of it, confirming the finding that an *ialB* mutant in *B. tribocorum* does not develop bacteremia in the rat-infection model (Christian Gille, unpublished data). The encoded inner membrane protein is involved in binding of *B. bacilliformis* to human erythrocytes *in vitro* (14); nevertheless, the molecular action of erythrocyte binding and invasion is still not elucidated. A further mutant was identified as *surA*, the first gene of a multifunctional operon that maintains its structure throughout the

proteobacteria. When the gene is disrupted in *Salmonella typhimurium*, bacteria are attenuated in mice (85). The same can be observed in an STM screen in *Klebsiella pneumoniae* during colonization of the gastrointestinal or urogenital tract of mice (83). SurA of *E. coli* is a periplasmic molecular chaperone that assists correct folding of outer membrane proteins (39). The downstream gene *pdxA* is required for the biosynthesis of the cofactor pyridoxal phosphate. Insertional mutation of *surA* in *E. coli* leads to pyridoxal phosphate-auxotrophy, showing the polar effect of this mutation and, thus, the operon organization (88). Further studies on *Bartonella* could provide evidence whether disturbing the regulation of stationary-phase survival by the folding action of SurA or the cofactor auxotrophy generated the abacteremic phenotype.

Transport

Transport processes also contribute to the interaction with the environment and are of prime importance for bacteria. In pathogenic bacteria, nutritional uptake, adhesion to the substratum, intercellular communication and interaction, or environmental sensing all contribute to the bacterial ability to establish infection and to persist in their appropriate host. Two clusters of genes with transport function code for the two T4SS of *B. tribocorum*, which are essential for *in vivo* pathogenesis (71, 74). Our screen revealed eleven mutants carrying transposon insertions in the *virB/D4* cluster (*virB2, virB4, virB5, virB6, virB9, virB10, virD4*) and twelve insertions in the *trw* cluster (*trwE, trwF, trwJ4, trwL6, trwL7, trwM,* and upstream of *trwL5* and *trwL6*). This firstly showed that already known pathogenicity factors of *Bartonella* can be re-identified in large number. Secondly, it indicated that more than only the already tested T4SS mutants (*virB4, virB10, virD4; trwD, trwE*) are essential for correct functioning of the T4SS apparatuses, though polar effects on downstream genes, due to the transposon insertion in an operon, would have to be excluded experimentally. T4SS serve as molecular injection devices directly injecting bacterial effector proteins into the host cell cytoplasm. It was shown in *B. henselae* that the effector proteins of the *virB/D4* T4SS, encoded by seven *bep* genes (*bepA-G*), are responsible for the prominent changes induced in endothelial cells *in vitro* (72). Surprisingly, only one of these seven *bep* genes (*bepD1*) contained a mutation that led to a abacteremic phenotype. Type III secretion systems (T3SS) are functionally similar to T4SS. Interestingly, in an STM screen of *Yersinia enterocolitica*-systemic infection in mice, several genes of the T3SS machinery were shown to be essential, but YopP is the only of six described T3SS effectors identified essential (18), similar to the finding in *Bartonella* type IV

secretion. The phenomenon of *bepD1* we investigated further with a new approach and this will be discussed later in this chapter.

Iron is an essential cofactor for cell metabolism and bacteria are competing with host cells for this factor. Therefore, efficient iron uptake from a hostile environment is another vital process in pathogenic bacteria. In contrast, sequestering free iron by iron-binding proteins, like transferrin, lactoferrin, or ferritin, constitutes a host defense mechanism against invading microorganisms (recently reviewed by Schaible and Kaufmann [68]). We discovered ten abacteremic mutants with mutations in seven genes coding for at least three different systems involved in iron binding and uptake. The affected hemin-binding protein B (HbpB) was shown in *B. quintana* to belong to a family of outer membrane proteins involved in hemin binding (51). Bartonellae are strictly dependent on hemin for growth, which was shown for *B. henselae* (66) and for *B. quintana* (52). Strikingly, the most abundant Hbp in *B. quintana*, namely HbpA, could be mutated by site-directed mutagenesis without impairing growth (51). The difference of HbpB to all other members of the Hbp family in *B. quintana* is a large 510-bp insert with tandem repeats contained in one extracellular loop (51). HbpB of *B. tribocorum* contains an even larger insert of 790 bp, but of different composition. We speculate that this extended extracellular loop contributes to the hemin-binding function, which cannot be complemented by other family members in contrast to HbpA. Typically, hemin uptake is a TonB-dependent process involving an ABC transporter (40). The *hutABC* locus encodes such a transporter and the *hutA* gene carried three, the *hutC* gene two transposon insertions. Also two genes for the TonB energizer system, *tonB* and *exbB*, were affected among the abacteremic mutants. This energizer system seems to be important for pathogenic bacteria in various environments, since it was identified in several STM screens like e.g. in a screen of systemic rat infection by *N. meningitidis* (84), of gastrointestinal mouse colonization by *Vibrio cholerae* (50), and of pneumonal pig infection by *Actinobacillus pleuropneumoniae* (77). Additionally, two genes of unlinked iron-uptake systems, also belonging to the ABC transporter family, *ceuD* and *yfeD*, contained insertions. This shows that at least three different iron-uptake systems are active and individually essential during infection of the mammalian host. The question remains, whether these systems are all active at the same time independently or switched on and off in different niches in the host.

One of the abacteremic mutants contained the insertion inside *secB*, whose gene product binds nonfolded proteins. Due to specific binding of SecB to SecA, it plays a crucial role during protein export by delivering protein precursors to the general secretory pathway (GSP) apparatus (58). Many pathogenicity factors, including adhesins and invasins, are

secreted via the GSP (81). So, the abrogation of this central secretion function could be the reason for its detection in this STM screen. But SecB can also act as general nonspecific chaperone in the cytosolic protein pool, which was shown recently for *E. coli* (89), and is involved in GSP-independent secretion of a hemophore by *Serratia marcescens* through an ABC transporter (67). There is no similarity of this ABC transporter to those found in *Bartonella*; nevertheless, SecB seems to be involved in a broad spectrum of secretion processes and its deletion in *B. tribocorum* certainly affected one of these functions.

Amino acid uptake seems to be important for survival of several pathogens inside the host (41). Consistently, several amino acid transporters could be identified in the STM screen. Two belong to ABC transporters: the ATP-binding protein LivF (084H11), responsible for the uptake of leucine, isoleucine, and valine, and a CH coding for a periplasmic amino acid-binding protein affected in mutants 045B05 and 049F11. CycA is described in *E. coli* as H^+-symporter responsible for the uptake of D-serine, D-alanine, and glycine (62). In α-proteobacteria, *cycA* is only present in the genus *Bartonella* and in the closely related genus *Brucella*, but only in *B. tribocorum*, this gene is duplicated. Despite a high degree of identity (74% identical, 86% similar amino acids), cross-complementation was not possible from CycA2 to CycA1. A mutant in *cycA2* exhibited an outcompeted phenotype, which could be due to weak attenuation caused by the mutation or partial complementation from CycA1 to CycA2. Since gene duplication may lead to new virulence factors, the elucidation of the individual functions of CycA1 and CycA2 will be very interesting. The *pha* operon (with a transposon insertion, upstream of its first gene *phaA*) mediates osmotolerance in *Sinorhizobium meliloti* by its K^+-efflux activity, which is important during plant cell invasion (57). The operon structure is conserved in the *Bartonella* species, indicating a possible role of this transporter for adaptation to new host niches. All other found transporters belong to the ABC transporter family, either mediating glycerol-3-phosphate uptake (*ugpA* and *ugpB*, see below), or transport of presently unknown substrates (*yhjE*, which is affected three times independently, and two CH proteins affected in mutants 023C11 and 045E11).

Gene-expression regulation

The ability to modulate gene expression to adapt to new environments during interaction with different niches is also a key factor of bacterial pathogenicity. One class of regulators responding to extracellular stimuli is constituted by the two-component regulatory systems (TCRS). They are typically composed of a sensor histidine kinase, spanning the bacterial cytoplasma membrane, and a cytoplasmic response regulator acting as transcription factor. In this screen, we discovered a TCRS with BatS as sensor kinase and BatR as response regulator. This BatR/BatS system is highly conserved in the α-proteobacteria. In *Agrobacterium tumefaciens,* the homologous ChvG/ChvI system is necessary for bacteria-induced tumor formation in plants and is a regulator of acid-inducible genes, like e.g. the VirB T4SS (46). The ExoS/ChvI system of *S. meliloti* is essential for endosymbiosis (9), and the BvrS/BvrR system of *Brucella spp.* for intracellular survival and the expression of outer membrane proteins (27, 80). Transcriptional profiling in *B. henselae* discovered the BatS/BatR system as the key regulator of the VirB/D4 T4SS and of several outer membrane proteins (M. Dehio, A. Basler, M. Quebatte, G. Raddatz, S. G. Andersson, and C. Dehio, unpublished results). These results all support the notion that the BatS/BatR regulatory system plays a central role in *Bartonella* pathogenesis. A second TCRS, identified in the STM screen, was the FeuQ/FeuP system with two transposon insertions in *feuQ* and none in *feuP*. Its ortholog in *Rhizobium leguminosarum* is required for the acquisition of iron shown by site-directed mutagenesis in *feuQ* (mutagenesis was not achievable in *feuP*), but the precise molecular mechanism is unknown (90). Interestingly, the orthologous system in *Brucella suis* is not important for survival in marcrophages, *in vitro* growth under iron limitation, or *in vivo* growth in a murine model (24). Since the *feuP* response regulator orthologs exhibit high degree of amino acid identity (between 69-77% for *Bartonella, Brucella,* and *Rhizobium*), the differences in the *feuQ* sensor kinase orthologs (between 41-50% amino acid identity for *Bartonella, Brucella,* and *Rhizobium*) may reflect differences in regulation responding to a different stimulus. A further indication for differences in regulation by the FeuQ/P system is the fact that *Rhizobium* and *Brucella* rely mainly on self-produced siderophores to scavenge iron from host iron-binding molecules, whereas *Bartonella* rather directly binds hemin of the host (see above). We identified eleven further regulatory genes containing transposon insertions, where six are associated with virulence in other pathogens (*chvD* [47] and *ros* [15] in *A. tumefaciens, gidA* in *Aeromonas hydrophila* [76], *kpsF* e.g. in *E. coli* K1 [12], *spoT* e.g. in *Mycobacterium tuberculosis* [16], and *lepA* in *Legionella pneumophila* [8]). KpsF is one of many proteins involved in the production of a polysaccharide capsule described for *E. coli* K1 and *S. meliloti*

(12, 63). KpsF functions as an accessory or regulatory component of the export translocase (12). Since *Bartonella* does not contain the genes for the biosynthesis of the capsule, the regulatory function of KpsF could influence different transporters. SpoT is a bifunctional enzyme (also called Rel, mainly in Gram-positive bacteria) responsible for the synthesis and degradation of the signal molecule guanosine 3', 5'-bispyrophosphate (ppGpp). The synthesis of this molecule is induced under nutrient-limited conditions, which then changes transport, metabolism of carbohydrates, amino acids, and phospholipids, known as the stringent response (54). In *M. tuberculosis*, the SpoT homolog Rel is necessary for adaptation to stationary phase in long-term persistence in mice (16). *Bartonella* also has to adopt a non-replicative state inside erythrocytes, when nutrients and space are limiting, which resembles stationary phase. It will be interesting to investigate, whether this change in behavior depends on the stringent response. LepA is a universal bacterial GTPase, which might regulate ribosome function (5). Though shown not to be essential for virulence, in *L. pneumophila*, LepA is translocated via the Icm/Dot T4SS into the cytosol of protozoa (8). Since the LepA protein of *B. tribocorum* is highly homologous to LepA of *E. coli* (58% identity, 75% similarity) and of *L. pneumophila* (60% identity, 77% similarity), this GTPase might be a T4SS substrate in *Bartonella*, although there was no clear indication for a possible *Bartonella* intercellular delivery domain, typical for VirB/D4 effector proteins (72). As translocated effector, LepA might exert its GTPase activity in target cells. This is further supported by the finding that LepA of *B. tribocorum* shares 45% identity and 65% similarity with a *Saccharomyces cerevisiae* GTPase of unknown function called GUF1 (36). This screen further identified four regulatory genes before not been associated with pathogenesis. HflKC constitute a membrane-anchored regulatory complex in *E. coli*, which interacts with the FtsH zinc-metalloprotease involved in selective degradation of membrane proteins, if they are uncomplexed (e.g. SecY of GSP, F_0 of H^+-ATPase) or unstable cytosolic regulatory proteins (34). *Bartonella* contains an orthologous *hflKC* locus (with *hflK* affected in mutant 035F08, and *hflC* with an outcompeted phenotype) and a homolog of FtsH. PrmC, formerly named HemK, is a warning sign to not only rely on sequence homology in gene annotation (13): most *hemK* genes are annotated as protoporphyrinogen oxidase in heme biosynthesis, whereas no such activity could be experimentally proven. Instead PrmC/HemK in *E. coli* methylates a glutamine residue to yield N-5-methylglutamine in translational release factors, possibly regulating protein translation (53). Heurgue-Hamard et al. proposed therefore the new name PrmC (31). The other three putative regulators, whose genes were affected by the mutants

065D10, 074E05, and 084C04, are CH proteins with so far no indication for a specific function.

Cell envelope integrity

Bacterial proteins with a function in maintaining cell envelope integrity certainly are also important pathogenicity factors. All of the genes with deleterious transposon insertions belonging to this category (*pal*, *tolQ*, *ispZ*, *phoH*, and a mutation upstream of *gpsA*) code for membrane or periplasmic proteins. Pal and TolQ belong to the Tol-Pal system, shown in *E. coli* to maintain outer membrane integrity (6). *Bartonella* species have this system organized syntenically to *E. coli*, indicating a functional conservation of the system. IspZ is a putative intracellular septation protein in some α-proteobacteria, also sharing homology to IspZ of γ-proteobacteria (e.g. *Yersinia pestis* or *S. marcescens*), but no experiments were published on this protein. The genes of PhoH and GpsA are both members of the phosphate starvation-inducible (*psi*) genes in *E. coli* (11, 35) as are also the *ugp* transporter genes (see above). Ugp transports glycerol-3-phosphate, a precursor for phospholipid biosynthesis. While for PhoH only bioinformatical data point in this direction (32), the importance of GpsA in phospholipid biosynthesis is proven experimentally (59). The major regulatory molecule tightly regulating this process is ppGpp (78), which is synthesized and degraded by SpoT (see above). All these key factors involved in phospholipid biosynthesis were affected by transposon mutation in *B. tribocorum*, firstly indicating a similar regulation circuit in *Bartonella* and secondly highlighting the importance of this process *in vivo*.

Metabolism

The nutritional environment of the host cell imposes a requirement for *de novo* biosynthesis of various amino acids, cofactors, and nucleotides in many pathogens. Since the requirements change in different niches of the host, important insights into the different nutritional constrictions inside the host can be gained by the analysis of auxotrophic mutants, which can grow on complex medium *in vitro*, but are not capable to survive in nutritionally deprived niches in the host. We distinguished here between involvement in energy metabolism, amino acid or cofactor biosynthesis, carbon metabolism, and DNA metabolism and modification. PncA is active in energy metabolism in *E. coli* being a nicotinamidase in the cyclical salvage pathway for the production of NAD from nicotinamide (55). The genes *aroE, dhs, glyA*, and *proA* are involved in amino acid biosynthesis in *E. coli*. The genes *aroE* and *aroF* (called *dhs* in *A. tumefaciens*) participate in chorismate biosynthesis, required for the synthesis of

aromatic amino acids; *glyA*, coding for serine hydroxymethyltransferase (SHMT, see also below), is a key enzyme for the biosynthesis of glycine, methionine, purines, thymidine, choline, and lipids, and is proven essential for symbiosis of *Bradyrhizobium japonicum* (64); proline biosynthesis depends on *proA* (23). In *E. coli* cofactor biosynthesis, *cobT* (upstream intergenic hit in 061E02) is involved in cobalamin biosynthesis, *panB* in pantothenate biosynthesis, important for the assembly of coenzyme A, *ribD* in riboflavin (FMN, FAD) biosynthesis, and *ubiA* in ubiquinone biosynthesis (for an overview over all the pathways, see [54] or www.biocyc.org). All these auxotrophies still await experimental testing of *B. tribocorum* growth on minimal medium deprived of the respective essential factor. MaeE catalyzes the oxidative carboxylation of malate to pyruvate, a key step in carbon metabolism. An enzyme with 5-formyltetrahydrofolate (formyl-THF) cyclo-ligase activity is widespread in bacteria and eukaryotes (normally found in mitochondria). It converts irreversibly formyl-THF, a potent inhibitor of SHMT (see *glyA* above) and other enzymes of C_1 metabolism, to 5,10-methenyl-THF. This conversion not only derepresses SHMT, 5,10-methenyl-THF itself is a substrate for SHMT in C_1 metabolism (82). The genes involved in this pathway, *glyA* (see above) and the cyclo-ligase gene (in mutant 010H04), were discovered in the STM screen, indicating the importance of C_1 metabolism for pathogenesis. Furthermore, DNA modification enzymes like RecA and UvrB, active in DNA repair under stress conditions, are important for pathogens, because the situation in the host elicits major stress for invading bacteria. The importance of RecA *in vivo* has already been shown in *Brucella abortus* (86) and the expression of *uvrB* was shown in *E. coli* to be indirectly controlled by RecA (69). In *Bartonella*, stress conditions may include survival in macrophages (33), an acidified environment, in which *uvrB* was shown to be important for survival of *M. tuberculosis* (19).

Unknown function

For 19 genes discovered in this STM screen, we do not have good indications for their functional role in the bacterial cell. Five genes contain predicted signal peptide sequences at their N-terminus, indicating that their gene products are secreted. We predicted membrane localization for five gene products because of the presence of transmembrane helices. Of special interest is a membrane protein (affected in 045H05) with a GGDEF and an EAL domain. These two domains are described to inversely regulate the levels of cyclic di-GMP, a second messenger involved in the regulation of many cellular processes (reviewed by D'Argenio and Miller [17]). Normally, bacteria contain more than one protein containing one or both domains, but in *Bartonella*, only this one protein was found. If its enzymatic action on

cyclic di-GMP can be shown, it would be an ideal system to study changes in cyclic di-GMP levels. Two genes affected by transposon insertions (069G06, 069H03) have no homologs in the two fully sequenced *Bartonella* genomes and seem to be of phage origin. Closer analysis of the phage loci in *B. tribocorum* may clarify the role of these two proteins. Of highest interest is one large *Bartonella*-specific protein (approx. 1501 aa), whose gene contained five independent transposon insertions. It carries a predicted signal peptide, but beyond this, no homology to other proteins available to date and no obvious domain structure could be observed. The orthologous regions in *B. henselae* and *B. quintana*, with proteins annotated as surface proteins, show similarity and synteny much below the normal level.
[The remainder of 13 mutants can only certainly be assigned to an insertion site and affected ORF, when the assembly is finished.]

Proof of non-essentiality of individual *bep* genes in *B. tribocorum*

To our best knowledge, no previous STM study could answer, why some presumed pathogenicity factors did not show up in the screen. Is it because this factor is individually not essential in pathogenesis, because it can be complemented *in trans* by co-infecting wild-type like bacteria, or because there is no mutant in the STM library with its gene disrupted? We consider this an important issue in an STM study, since it provides information about the quality of the library, the number of disrupted genes, and the number of disruptions per gene. One approach published by Geoffroy et al. could ultimately answer this question by identifiying the transposon insertion sites of all mutants tested in their STM screen (26), but this is a very time- and labor-consuming strategy. So, we established a more focused approach, similar to the genetic footprinting strategy described by Smith et al. (79), to answer the question stated above without identifying all transposon insertion sites. This PCR-based approach discovers transposon insertions in a selected gene scanning the whole mutant library.

As an examplary region to test this strategy, we chose the *virB/D4/bep* cluster, because of the obvious imbalance of abacteremic mutants obtained by STM (see Figure 4B). They are almost exclusively present in the *virB/D4* genes, coding for the T4SS apparatus, and almost absent in the *bep* genes, coding for the exported VirB/D4 effector proteins (72). Our PCR screen verified all abacteremic mutants identified by STM in *virB8*-*virB11* and *virD4*. The absence of mutations in *virB8* and *virB11* could be explained by their small size, by insufficient coverage of the transposon library, or by a possible lethal mutant phenotype even under *in vitro* conditions. In contrast, we discovered ten mutations in four *bep* genes (Figure 4B, Table

2), which all did not show severe attenuation in the STM screen. We concluded that these *bep* genes were individually not essential for pathogenicity. To exclude that extracellular complementation by the *bep* orthologs from other mutant strains in the pool leads to mutant behavior like wild-type bacteria, we infected rats with single *bep* mutants (with or without competition with wild-type bacteria). These mutants grew and persisted inside the rats comparable to wild-type bacteria shown by CI of the mutants not differing more than ten-fold from wild-type bacteria (Table 2). This demonstrated that these tested *bep* genes were individually not essential in the *in vivo* situation or were intracellularly complementated by *bep* paralogs. Interestingly, the Bep proteins can be distinguished into three categories: BepA, BepB, and BepC show similar protein domain organization and reveal in *B. tribocorum* 40% identical amino acids compared to each other. Accordingly, BepD1, BepD2, BepE, and BepF constitute a second cluster of effector proteins, less similar in amino acid sequence, but each containing multiple putative tyrosine phosphorylation motifs. In *B. henselae,* BepD was shown to get tyrosine-phosphorylated upon translocation into eukaryotic target cells *in vitro* (72). The third category consists only of BepG, which is present in *B. henselae*, but not in *B. tribocorum*. The individual paralogs of one cluster might complement each other. The PCR screen could not identify mutants in some *bep* genes, namely *bepB, bepC,* and *bepE*, in the mutant library, but we do not want to make conclusive statements based on the absence of a PCR band; so, their contribution to pathogenicity remains to be elucidated. Only one *bepD1* mutant (of six *bepD1* mutants in total) showed an abacteremic phenotype in the STM screen. Whether this mutation has a polar effect on the downstream *bepD1D2EF* cluster or whether the mutant protein exerts a dominant negative effect during active protein secretion, e.g. by blocking the transport machinery, needs further experimental scrutiny. This PCR-screening approach showed its suitability to analyze focally individual genes or gene clusters, enabling the comparison of the abacteremic mutants with chosen mutants from the whole mutant library used in the screen. Additionally, this approach is a fast way to retrieve a transposon insertion mutant in a gene of interest from the library for further testing.

Conclusions

Summarizing the results of the STM screen, we conclude that a major part of the affected genes code for proteins involved in cellular interaction with the host or gene-expression regulation, where future experiments have to reveal the specific contribution to pathogenesis. Remarkably, almost all discovered pathogenicity factors have close homologs in the related α-proteobacteria, confirming earlier observations, (i) that plant- and human-pathogenic α-proteobacteria share survival strategies and host-cell interaction schemes (2) and (ii) that genes required in the intracellular lifestyle of *Brucella* are also involved in *A. tumefaciens* virulence and *Rhizobium* symbiosis (44). The latter finding is reflected in the found overlap between this STM screen in *Bartonella* and those in *Brucella* (22). Besides these pathogenicity factors with a known or presumed role in pathogenesis, we also identified a large number of CH and hypothetical proteins, supporting the notion of Perry (56) that so far, we have only a very limited understanding of the many gene products found in the bacterial genomes sequenced to date. We anticipate that this large-scale STM analysis and future work based upon it will lead to a deeper understanding of the processes by which *B. tribocorum* establishes a successful infection and maintains its persistence inside the host. Comparative analyses with the other bartonellae will broaden this knowledge to understand the common features of *Bartonella* pathogenesis.

MATERIAL AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 3. For cloning, *E. coli* NovaBlue was used, and for plasmid mobilization to *B. tribocorum*, *E. coli* β2155. *E. coli* strains were grown at 37°C overnight in Luria Bertani broth supplemented with 200 mg l^{-1} ampicillin, 50 mg l^{-1} kanamycin, or 1 mM diaminopimelic acid, where appropriate. *B. tribocorum* IBS 506^T and its derivatives were grown for 2 days on Columbia agar containing 5% defibrinated sheep blood (CBA) in a humidified atmosphere with 5% CO_2 at 35°C. For the *B. tribocorum* transposon mutants, CBA was supplemented with 50 mg l^{-1} kanamycin at all times (CBA-Km). For the Sm^r *B. tribocorum* strain RSE149, CBA was supplemented with 100 mg l^{-1} streptomycin at all times (CBA-Sm).

Animal husbandry and housing. RCC Füllinsdorf, Switzerland, provided 10 weeks-old female WISTAR rats, which were housed in an animal facility under Biosafety Level II conditions (2 rats per cage). All animals showed good health and did not exhibit any signs of disease or changed behavior before, during, and after the experiments. They could acclimate to the facility and the diet at least 5 days prior to infection. Food and water were provided *ad libitum*. Animal care was performed and animal well-being ensured in accordance with the Swiss Act on Animal Protection and Good Animal Care Practice.

Transposon vector construction. To construct the transposon vector pHS006, the following strategy was applied: The plasmid pHS001 was derived by digesting pLRS14 with *Xba*I/*Bam*HI followed by a Klenow fill-in reaction and self-ligation. A fragment of pTnT7PA containing the *Himar1* transposon and the oriV was cut out of the plasmid with *Nde*I (4646 bp), treated with Klenow fill-in, cut again with *Pci*I, and ligated into pHS001, cut with *Nhe*I followed by Klenow fill-in and cutting with *Pci*I, giving rise to pHS002. The gene of the hyperactive transposase was isolated from pBADC9 by digestion with *Nhe*I/*Hind*III and Klenow fill-in (1133 bp). This fragment was then ligated into the *Ecl*136II-digested pHS002, resulting in pHS003 (Figure 1). The plasmid pHS005 was derived from pHS003 by killing of a second *Xho*I site (partial digestion with *Xho*I, Klenow fill-in reaction, self-ligation), additional to the *Xho*I site in the transposon. The plasmid pHS006 was derived from pHS005 by killing of a second *Aat*II site (partial digestion with *Xho*I, Klenow exonuclease reaction, self-ligation), additional to the *Aat*II site in the transposon.

Cloning and transposition test. Plasmid pHS003 was transferred from *E. coli* NovaBlue to *E. coli* β2155 by electroporation and subsequently to *B. tribocorum* by twoparental mating (21). Thirteen single kanamycin-resistant *B. tribocorum* colonies were isolated and, firstly, tested by colony PCR for the presence of the transposon or of the whole vector using the primers LHS004/005 (Table 4) or LHS003/007 on the transposon or in the vector backbone, respectively. Secondly, southern blot analysis was done with these 13 samples. Therefore, genomic DNA of the mutants was isolated with the QIAGEN Genomic DNA Isolation kit (QIAGEN, Hilden, Germany), digested with EcoRI, separated by agarose gel electrophorese, and blotted on a nylon membrane. The membrane was probed using a 1216 bp *Xba*I/*Xho*I fragment of pHS003 as transposon probe and a 774 bp *Pst*I fragment of pHS003 as vector backbone probe following standard protocols (65).

Tag construction and insertion into the vector. A pool of degenerated single-stranded 120-bp DNA molecules (STM oligo) containing a central stretch of 50 random bp ($[NK]_{50}$) flanked by two constant sequences was generated by oligonucleotide synthesis (Microsynth, Balgach, Switzerland). These constant sequences contained *Aat*II and *Xho*I cutting sites directly flanking the random sequence. The STM oligo was amplified by PCR using primers LHS028 and LHS029, the resulting product recovered from a 3% agarose gel, digested with *Aat*II and *Xho*I, and subcloned into pHS006 (also digested with *Aat*II and *Xho*I). After introduction of tagged pHS006 into *E. coli* NovaBlue by electroporation, 96 single colonies were grown to larger quantity, plasmid DNA was extracted and sequenced with primer LHS018 and LHS019 to obtain the inserted tag sequence. From these sequenced plasmids, 59 contained a tag, were therefore chosen and denominated pHS006tag*n* (*n* stands for the tag number 001 to 096 according to the colony number from above). The corresponding *E. coli* strains were stored at -70°C.

Transposon library construction. A total of 42 pHS006tag*n* vectors were separately introduced into *E. coli* β2155 by electroporation and subsequently transferred to *B. tribocorum* by twoparental mating (21). A total of 36 vectors were chosen based on reproducible detection (see "Testing of tag-specific detection primers") and used to produce kanamycin-resistant *B. tribocorum* transconjugants. From each mating, 96 single kanamycin-resistant *B. tribocorum* colonies were transferred to a 96-well plate, labeled with the tag number for storage at -70°C.

For some mutants, presence of the transposon or of the whole vector was checked by PCR (see "Cloning and transposition test").

Testing of tag-specific detection primers. For each tag, a specific primer was designed (Table 4) for PCR together with a generic primer on the transposon (Srev01). Each primer was tested on a mix of all possible templates (as used in the input pools, see "PCR detection") and on this mix without the corresponding tag as template. If a primer was not specific, a new one was designed for this tag and retested. All 36 tags used in this study could be specifically and reproducibly detected.

Animal infections. Infections were done using the *B. tribocorum* rat-infection model described before (73). For infection, 36 differently tagged mutants were grown separately from the transposon library for each input pool, same amounts of each mutant were pooled in PBS directly before infection, and used to infect two rats with an inoculum of 10^9 bacteria (0.3 ml of OD_{595} = 1) intravenously in the tail vein. Half the remainder of these input pools was stored at -70°C, the other half was heat-denaturated at 100°C for 15 min and used as template for the detection PCR. Blood was taken from the tail vein of the infected rats after 7 and 14 days postinfection, bacteria released from erythrocytes by freeze-lysation, serially diluted in PBS, and plated on CBA-Km. Grown bacterial colonies (the output pool) were counted, harvested in PBS, one half stored at -70°C and the other half heat-denaturated at 100°C for 15 min. The latter part of the output pool served as template for the detection PCR. The rescreen was done following the same protocol with minor changes mentioned in the "Results". An in-pool CI of abacteremic mutants can be estimated using the determined CFU of wild-type bacteria after 7 or 14 days of infection (approx. 10^7 CFU per ml blood) and the detection limit of the detection PCR (approx. 10^2 CFU per ml blood) for calculation. The input ratio between a wild-type (or wild-type like) strain and an abacteremic mutant strain equals 1:1, so the in-pool CI can be calculated by dividing the CFU of the abacteremic mutant (below detection level) by the CFU of wild-type bacteria, which results in an in-pool CI of <0.00001.

PCR detection. For each input and output pool 36 tag-specific PCR reactions were done. For the PCR, the tag-specific primers (Table 4) were used together with the generic primer Srev01 to yield a product of approx. 600 bp. The conditions for the touchdown PCR were as follows: After a first denaturation step at 95°C for 5 min, 30 cycles of PCR followed.

The first 20 cycles consisted of denaturation at 95°C for 1 min, annealing for 30 s, that started with 62°C lowered in steps of 0.5°C every cycle, and extension at 72°C for 1 min. Then 10 cycles followed, with denaturation at 95°C for 1 min, annealing at 52°C for 30 s, and extension at 72°C for 1 min. The program was finished by a last extension step at 72°C for 5 min. The PCR products were displayed on a 1.7% agarose gel.

Identification and analysis of transposon insertion sites. Genomic DNA from single mutants, regrown from the mutant library, was isolated following the QIAGEN Genomic DNA Isolation protocol. Diluted genomic DNA (0.5 mg ml^{-1}) was used for sequencing with primers LHS001 and LHS002. The sequences obtained by the genomic sequencing were compared to the contigs of the *B. tribocorum* genome by BlastN to find the exact transposon insertion sites. In the surrounding of these sites, ORFs were predicted, and their translated amino acid sequence was compared to the nr database (http://www.ncbi.nlm.nih.gov/blast/html/blastcgihelp.html#protein_databases) by BlastP. For further analysis, the following programs or online-tools besides the ones on the NCBI-site were used: ClustalW (v1.82) (87) for multiple alignments, tRNAscan-SE (v1.21) (48) for prediction of tRNA genes, PSORTb (v2.0.3) (25) for subcellular protein localization, SMART (v4.0) (45) for protein domain comparisons, SignalP (v3.0) (4) for prediction of signal peptide sequences, the EMBOSS tools (60) for local sequence alignments or sequence modifications, the Biocyc database (http://www.biocyc.org, see also Krieger et al. [37]) for data searches about functional information of genes, coliBASE (7) and its derivative for rhizobacteria, RhizoDB, for ortholog searches or comparisons of synteny in γ- and α-proteobacteria, respectively.

PCR assay for further transposon insertions. For each tested gene, two primers were designed (Table 4), one shortly before the start codon (start primer) and the other one shortly after the stop codon (stop primer). The 96 heat-denaturated input pools served as template for PCR. For each gene, two 96-well PCR reactions were done on this template using the touchdown PCR program stated above. One PCR reaction with the gene start primer and the transposon-specific primer LHS018, and the other reaction with the gene stop primer and LHS018. Each PCR product was confirmed by a single PCR reaction on the respective input pool and used for sequencing with primer LHS018 for the signature-tag sequence, and primer LHS002 for the precise insertion site of the transposon.

Single and competitive infections with transposon insertion mutants in *bep* genes. Single mutants were grown on CBA-Km, harvested, and used to infect two rats with an inoculum of 10^9 bacteria (0.3 ml of OD_{595} = 1) intravenously in the tail vein. For competitional infections, the same amount of a single mutant and *B. tribocorum* RSE149 (grown on CBA-Sm) was mixed, adjusted to OD_{595} = 1, and used to infect another two rats as mentioned before with 0.3 ml of the adjusted bacterial suspension. Blood was taken from the tail vein of the infected rats after 7 and 14 days postinfection, measured by weight, bacteria released from erythrocytes by freeze-lysation, serially diluted in PBS, and plated on CBA-Km and on CBA-Sm. Grown bacterial colonies were counted and the CFU per ml blood was determined. CI were calculated by dividing the CFU of mutant bacteria by the CFU of wild-type bacteria.

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FIGURES AND TABLES

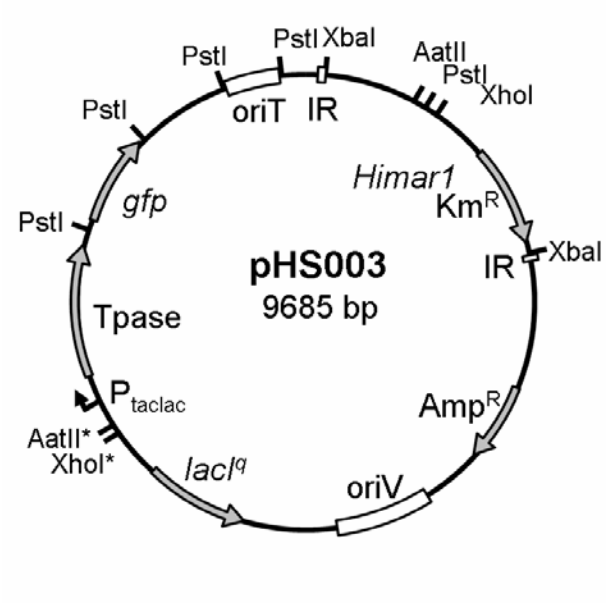

Figure 1: Plasmid map of the conjugative suicide mutagenesis vector pHS003. The plasmid contains the *Himar1* transposon, flanked by two inverted repeats (IR), the transposase (Tpase) under the control of the promoter P_{taclac}, followed by the *gfp* gene, the *lac* repressor (*lacI*^{q}), two antibiotic resistance markers indicated by Km^R (kanamycin) and Amp^R (ampicillin), the origin of replication (oriV) and the origin of conjugal transfer (oriT). Plasmid pHS006 is a derivative of pHS003 deleted for the two cutting sites marked with an asterisk. The tags were inserted into pHS006 between the remaining AatII and XhoI sites. For southern blot analysis, a XbaI/XhoI fragment containing Km^R was used as transposon probe and a PstI fragment containing *gfp*.

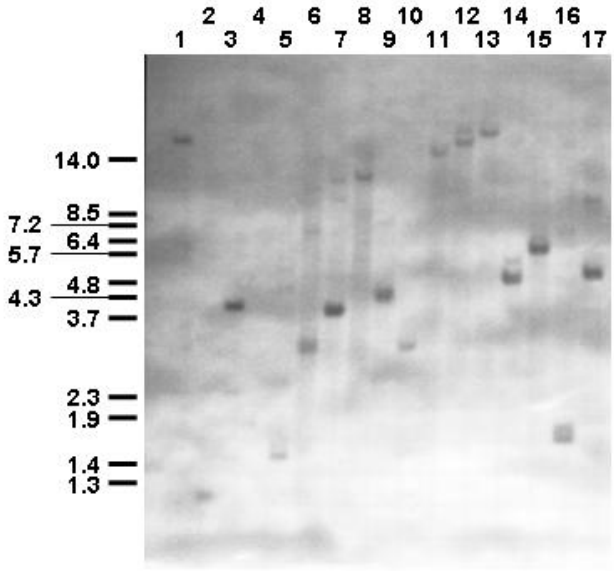

Figure 2 : Southern blot of single transposon insertion mutants. For southern blot analysis, a 1216 bp-XbaI/XhoI fragment of pHS003 (Figure 1) was used as transposon probe and a 774 bp-PstI fragment as vector backbone probe. The lanes are numbered from 1-17: 1, undigested pHS003; 2, pHS003 digested with XhoI/XbaI to yield the 774 bp-transposon probe fragment; 3, pHS003 digested with EcoRI to yield a 4156 bp-fragment; 4, *B. tribocorum* wild-type genomic DNA digested with EcoRI; 5-17, genomic DNA of 13 randomly chosen transpositional mutants, digested with EcoRI. Numbers on the left indicate the sizes in kbp.

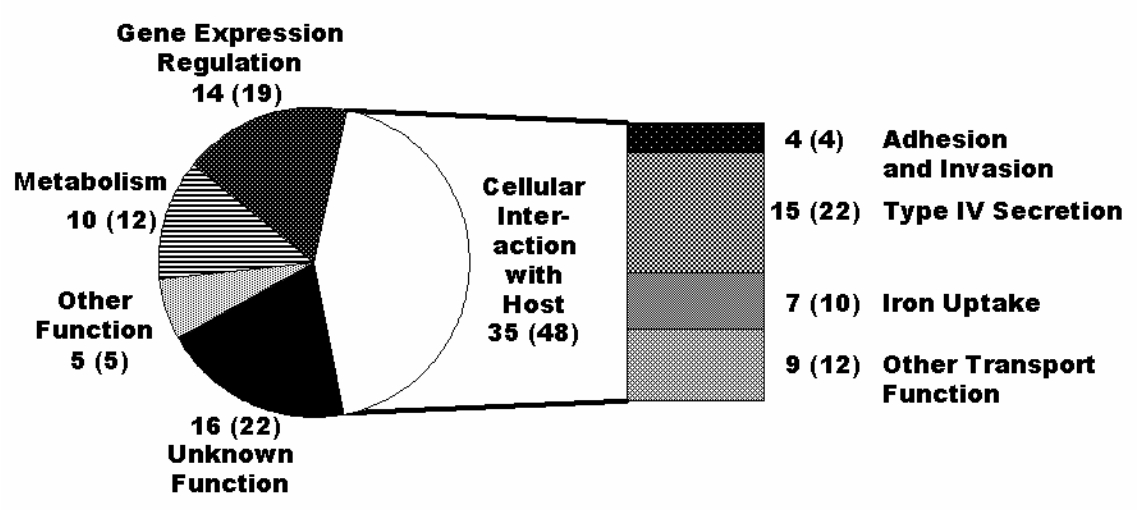

Figure 3: Functional classification of the STM mutants. The category "Cellular Interaction with Host" is subdivided in the right panel. In the different classes, numbers in front of brackets indicate the distinct affected genes; numbers in brackets indicate the number of independent transposon insertion mutants.

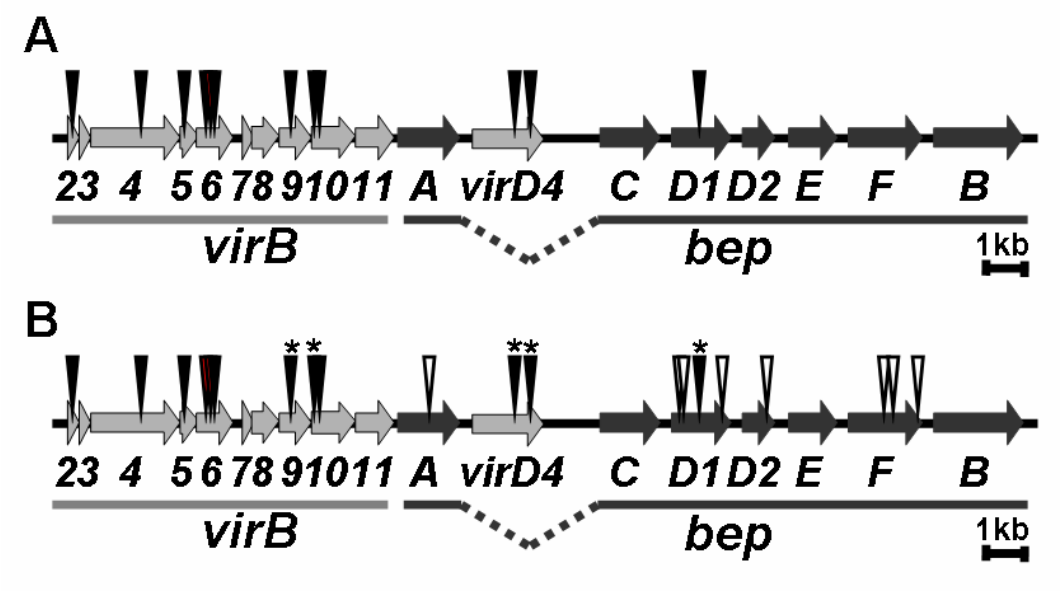

Figure 4: Mapping of transposon insertions in the *virB/D4/bep* cluster. The screen for abacteremic mutant phenotypes revealed a high frequency of insertions in the structural genes of the T4SS (*virB* and *virD4*) and only one insertion in one of the effectors (*bepD1*) (A). Other effector genes also contained transposon insertions not leading to a abacteremic phenotype shown by testing the mutant library (B). The filled triangles symbolize the transposon insertions generating abacteremic mutants (asterisks indicate insertions confirmed by the insertional analysis using the PCR assay), the empty triangles those insertions identified by the PCR assay.

Table 1: Characterization of the abacteremic mutants. Gene Size is given in base pairs (bp), the columns BH and BQ list the homologous genes in *B. henselae* and *B. quintana*, respectively. The last column lists the number of animals, the mutant was tested in. CH denotes conserved hypothetical proteins.

Gene Name	Mutant	Putative Function	Gene Size	BH	BQ	Animals
Cellular interaction with host						
Adhesion and invasion						
ibaB	087G03	inducible *Bartonella* autotransporter	2475	BH13140	BH13160	6
ibaC	035E04	inducible *Bartonella* autotransporter	2421	BH13160	BH13140	6
ibaD	035B11	inducible *Bartonella* autotransporter	1656	BH13120	BH13160	6
badA[a]	087E05	adhesin	15168	BH01510	BQ01400	6
ialB/invB[a]	079G06	invasion protein B	558	BH01650	BQ01550	6
ialB/invB	025H06	invasion protein B	558	BH01650	BQ01550	6
surA[a]	087D02	assembly of outer membrane proteins	966	BH05420	BQ04600	6
Type IV secretion						
trwE	041H01	channel subunit	1140	BH15750	BQ12670	6
trwE	069G08	channel subunit	1140	BH15750	BQ12670	6
trwF	025D10	ATPase energizer	825	BH15740	BQ12660	6
trwF	086D01	ATPase energizer	825	BH15740	BQ12660	6
trwJ4	069B11	pilus subunit	735	BH15670	BQ12590	6
trwL2	025E03	pilin subunit	318	BH15630	BQ12530	6
trwL5[a]	061H11	pilin subunit	312	BH15600	BQ12530	6
trwL6[a]	084H03	pilin subunit	318	BH15580	BQ12530	6
trwL6	068E08	pilin subunit	318	BH15580	BQ12530	14
trwL6	083C05	pilin subunit	318	BH15580	BQ12530	10
trwL7	065C10	pilin subunit	309	BH15640	BQ12560	6
trwM	048F08	outer membrane subunit	306	BH15650	BQ12570	6
virB2	043H04	pilin subunit	318	BH13260	BQ10530	10
virB4	005G04	ATPase energizer	2352	BH13280	BQ10550	6
virB5	086H09	pilus subunit	483	BH13290	BQ10560	6
virB6	045C04	channel subunit	975	BH13300	BQ10570	6
virB6	048C07	channel subunit	975	BH13300	BQ10570	6
virB6	083H12	channel subunit	975	BH13300	BQ10570	6
virB9	023A04	outer membrane channel subunit	864	BH13330	BQ10600	10
virB10	035D10	channel subunit	1170	BH13340	BQ10610	6
virB10	079H12	channel subunit	1170	BH13340	BQ10610	6
virD4	045D05	coupling protein	1914	BH13380	BQ10640	6
virD4	048B01	coupling protein	1914	BH13380	BQ10640	6
bepD1	041H04	virB/D4 T4SS substrate	1572	BH13410	-	14
Iron uptake						
hbpB	045C06	hemin-binding protein B	1761	BH02570	BQ02430	6
hutA	065H09	outer membrane heme receptor	2175	BH04970	BQ04160	6
hutA	068G04	outer membrane heme receptor	2175	BH04970	BQ04160	14
hutA	086B05	outer membrane heme receptor	2175	BH04970	BQ04160	6
hutC	003C03	hemin transporter permease	1077	BH04940	BQ04130	10
hutC	041F11	hemin transporter permease	1077	BH04940	BQ04130	10
tonB	085H10	energizer protein for outer membrane transport	777	BH04980	BQ04170	6
exbB	049C05	biopolymer transport protein	873	BH14710	BQ11710	14
ceuD	025E09	ABC transporter, ATP-binding protein	756	-	BQ10330	6
yfeD	044A11	iron transport system membrane protein	903	BH00890	BQ00820	10
Other transport function						
secB	083C01	protein translocase	474	BH01020	BQ00950	14
livF	084H11	amino acid ABC transporter, ATP-binding protein	792	BH08250	BQ06330	10
CH 1	045B05	amino acid ABC transporter, periplasmic binding protein	759	BH06370	BQ06860	6
CH 1	049F11	amino acid ABC transporter, periplasmic binding protein	759	BH06370	BQ06860	10
cycA1	025T01[b]	D-alanine, D-serine, glycine transporter	1398	BH10880	BQ08530	6
phaA[a]	004F08	ds60 hypo immunodominant protein (B.suis) us180 phaA (potassium efflux transporter)	2919	BH16460	BQ13360	6
ugpA	004E12	glycerol-3-phosphate permease ABC protein	882	BH01860	BQ01750	6

Gene Name	Mutant	Putative Function	Gene Size	BH	BQ	Animals
Other transport function (continued)						
ugpB	048B12	glycerol-3-phosphate-binding periplasmic ABC protein	1332	BH01870	BQ01760	6
yhjE	045F01	ABC transporter, membrane spanning protein	1287	BH05480	BQ04660	6
yhjE	048A01	ABC transporter, membrane spanning protein	1287	BH05480	BQ04660	6
yhjE	079H06	ABC transporter, membrane spanning protein	1287	BH05480	BQ04660	6
CH	023C11	ABC transporter, permease	1980	BH15460	BQ12380	10
CH	045E11	ABC transporter, permease	825	BH01900	BQ01780	6
Gene-expression regulation						
Two-component regulatory systems						
batR	005B11	response regulator	720	BH00620	BQ00560	6
batR	068D01	response regulator	720	BH00620	BQ00560	14
batR	079G05	response regulator	720	BH00620	BQ00560	6
batR	079E10	response regulator	720	BH00620	BQ00560	6
batS	025F03	sensor histidine kinase	1782	BH00610	BQ00550	10
feuQ	085F09	sensor histidine kinase	1419	BH04720	BQ03920	10
feuQ	086F05	sensor histidine kinase	1419	BH04720	BQ03920	10
CH	065D10	sigma-dependent response regulator	1410	BH01780	BQ01670	10
Other regulators						
chvD	083E10	ABC transporter, ATP-binding protein	1647	BH11380	BQ09000	14
gidA	085D11	glucose-inhibited division protein	1866	BH16680	BQ13560	10
hflK	035F08	membrane regulator of protease	1167	BH10960	BQ08610	6
kpsF	086E05	polysialic capsule regulator	1002	BH01760	BQ01650	10
kpsF	087E04	polysialic capsule regulator	1002	BH01760	BQ01650	10
lepA	061E01	GTP-binding protein	1803	BH00710	BQ00640	10
prmC	086C10	translational regulator	717	BH01960	BQ01840	10
rosAR	044H11	Ros/MucR family transcriptional regulator	435	BH04610	BQ03800	6
spoT	005E10	GTP pyrophosphokinase	2241	BH05040	BQ04230	6
CH	074E05	putative regulator	1419	BH03800	BQ02810	18
CH	084C04	putative regulator (*araC* family)	534	BH14920	BQ11900	14
Cell envelope integrity						
pal	041H09	peptidoglycan-associated lipoprotein	588	BH14790	BQ11770	10
tolQ	065G06	energizer protein for outer membrane transport	744	BH14870	BQ11850	14
gpsA[a]	079E08	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	1014	BH01260	BQ01190	6
ispZ	025D03	intracellular septation protein Z	714	BH16630	BQ13510	6
Metabolism						
Energy metabolism						
pncA	004E11	nicotinamidase	606	BH11400	BQ09020	6
pncA	010G11	nicotinamidase	606	BH11400	BQ09020	10
Amino acid biosynthesis						
aroE	049A01	shikimate 5-dehydrogenase	870	BH00030	BQ00030	6
dhs	004F11	2-dehydro-3-deoxyphosphoheptonatealdolase	1416	BH06440	BQ06790	6
glyA	045A07	glycine/serine hydroxymethyltransferase	1329	BH07540	BQ05390	6
proA	045E05	gamma-glutamyl phosphate reductase	1254	BH01580	BQ01480	6
proA	004D05	gamma-glutamyl phosphate reductase	1254	BH01580	BQ01480	6
Cofactor biosynthesis						
cobT[a]	061E02	N(1)-alpha-phosphoribosyltransferase	1005	BH08100	bad	6
panB	044G12	3-methyl-2-oxobutanoate hydroxymethyltransferase	813	BH05130	BQ04320	10
ribD	083F12	riboflavin biosynthesis protein	1125	BH07560	BQ05410	10
ubiA	061F09	4-hydroxybenzoate octaprenyltransferase	480	BH07550	BQ05400	10
Carbon metabolism						
maeB	061C01	NADP-dependent malic enzyme	2298	BH01010	BQ00940	6
CH	010H04	5-formyltetrahydrofolate cyclo-ligase	609	BH14400	BQ11340	14
DNA metabolism and modification						
recA	005C02	DNA repair protein	1098	BH10230	BQ07950	6
uvrB	045A11	excinuclease	2229	BH11720	BQ09330	6

Gene Name	Mutant	Putative Function	Gene Size	BH	BQ	Animals
Unknown function						
ordL	086D04	oxidoreductase	1281	BH03900	BQ02920	6
phoH	085G02	phosphate starvation induced protein	1146	BH02250	BQ02130	6
rnd	048D11	ribonuclease D	1263	BH09800	BQ07560	6
CH 2	043B07	membrane protein	1053	BH11410	BQ09030	18
CH 2	045B08	membrane protein	1053	BH11410	BQ09030	6
CH 3	044A03	signal peptide protein	507	BH00190	BQ00180	10
CH 3	087E03	signal peptide protein	507	BH00190	BQ00180	6
CH	025D02		1677	BH15980	BQ12880	6
CH	035B05		771	BH08320	BQ06260	6
CH	045B10	membrane-associated zinc metalloprotease	1158	BH06270	BQ06960	6
CH	045H05	GGDEF/EAL domain membrane protein	2562	BH04460	BQ03650	6
CH	061F10	methyltransferase	942	BH00120	BQ00110	6
CH	087H02	signal peptide membrane protein	807	BH12260	BQ09640	6
CH[a]	035F01	miaB family protein	1224	BH00200	BQ00190	6
CH[a]	044B07	us730 purE us200 CH membrane protein	507/603	BH14180	BQ11210	10
CH[a]	048F04	us100 signal peptide membrane protein	801	BH11730	BQ03410	6
hypo	025G04		3150	BH03150	-	10
hypo	069G06	phage-related membrane protein	1743	-	-	14
hypo	069H03	phage-related methyltransferase	819	-	-	14
hypo[a]	005G07	us400 CH signal peptide protein	381	BH16390	BQ13290	6
hypo[a]	086G03	us3 hypo us 300 hypo	420	BH14480/90	BQ11460/80	6
***Bartonella*-specific genes**						
NOHO03	005B02		4503	-	-	10
NOHO03	023H03		4503	-	-	6
NOHO03	062E05		4503	-	-	22
NOHO03	079H04		4503	-	-	10
NOHO03	079H10		4503	-	-	10
Check, when assembly is finished						
ig08	045G03	us305 comM competence protein us40 surface antigen	1530/1962	BH00440/50	BQ00390/400	6
ig09	005D06	us70 orphan us300 ch	693/978	BH05300/-	BQ04480/-	6
ig09	045D01	us70 orphan us300 ch	693/978	BH05300/-	BQ04480/-	6
ig01	023A02			nd	nd	6
ig02	035A01			nd	nd	6
ig06	086H01			nd	nd	6
ig12	004D01			nd	nd	6
ig13	084G11			nd	nd	10
ig14	045E10			nd	nd	6
ig15	086D03			BH13810	BQ10930	6
ig16	060C12			nd	nd	10
NOHO02	043H09			-	-	14

[a] The transposon insertion site lies upstream of the denoted gene
[b] This mutant is taken from a test round (T01)

Table 2: Results from the insertional analysis by PCR.

Gene Name	Mutant	CI (d14) [a]	Screen Result[b]
virB9	023A04	n.d.	am
virB10	035D10	n.d.	am
virB10	079H12	n.d.	am
virD4	045D05	n.d.	am
virD4	048B01	n.d.	am
bepA	010F12	0.2	WT
bepD1	024C01	n.d.	WT
bepD1	043C09	n.d.	WT
bepD1	043G02	n.d.	WT
bepD1	043H08	n.d.	WT
bepD1	041H04	0.0000001	am
bepD1	079F11	3	WT
bepD2	085E02	0.2	Dis
bepF	010B11	5	WT
bepF	044D09	n.d.	WT
bepF	049E10	n.d.	WT

[a] Competitive index (CI) was calculated as CFU of mutant recovered from blood/CFU of wild type recovered from blood

[b] Phenopytes: am, abacteremic mutant; WT, wild-type; Dis, disappearing (present on day 7, absent on day 14)

Table 3: Bacterial strains and plasmids used in this study

	Genotype and relevant phenotype	**Source or reference**
Strains		
B. tribocorum IBS 506^T	Isolate from the blood of a wild rat (CIP 105476^T)	(28)
B. tribocorum RSE149	Spontaneous Sm^R strain of IBS 506^T	(71)
E. coli NovaBlue	*endA1 hsdR17* (rK12$^-$ mK12$^+$) *supE44thi-1 recA1 gyrA96 relA1 lacF* (*proA*$^+$ *proB*$^+$ *lacI*^{q}*Z DM15*::*Tn10* Tc^r	Novagen, Madison, USA
E. coli β2155	*thrB1004 pro thi strA hsdS lacZΔM15* (F' *lacZΔM15 lacI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm* (Erm^R) *pir*::RP4 [::*kan* (Km^R) from SM10]	(21)
Plasmids		
pLRS14#10	Contains oriV, oriT, the *virB4* and *gfp* genes under P_{taclac}, Km^R	Schulein, unpublished
pBADC9	Contains *Himar1* tpase[a] (with Q131R and E137K mutations) under ara_{BAD} promoter	(38)
pTnT7P	Contains *Himar1* transposon (with Km^R) and Ap^R	E.J. Rubin, unpublished
pHS001	Derivative of pLRS14#10 with deletion of *virB4* gene	This work
pHS002	Derivative of pHS001 with the *Himar1* transposon (with Km^R) and Ap^R of pTnT7P	This work
pHS003	Derivative of pHS002 with the tpase[a] of pBADC9 under P_{taclac}	This work
pHS005	Derivative of pHS003 with one *Xho*I site removed	This work
pHS006	Derivative of pHS005 with one *Aat*II site removed	This work

[a] tpase = transposase

Table 4: Primers used in this study

Primers	Sequence (from 5' to 3')	Purpose
For STM establishment		
LHS001	TGTTGTTCCAGTTTGTAATACG	Sequencing out of transposon
LHS002	TCTTCTGAGCGGGACTCTG	Sequencing out of transposon
LHS003	CCTGGGCTTTTGCTGGCC	Amplification of part from vector backbone
LHS004	GGTACCGAGGACGCGTCG	Amplification of part from transposon
LHS005	CCCCAGAGTCCCGCTCAG	Amplification of part from transposon
LHS007	GGCGTGGAGCATCTGGTCG	Amplification of part from vector backbone
LHS018	TGAAGACGAAAGGGCCTCG	Check of signature-tag insertion
LHS019	CTTGCGGCAGCGTGAAGC	Check of signature-tag insertion
LHS028	TACAAAAAAGCAGGCTGACGTC	Amplification of STMoligo
LHS029	ACAAGAAAGCTGGGTCTCGAG	Amplification of STMoligo
STMoligo	GGGGACAAGTTTGTACAAAAAAGCA GGCTGACGTC $[NK]_{25}$ CTCGAGACCCA GCTTTCTTGTACAAAGTGGTCCCC	Starting template for signature-tag generation
For STM detection		
S003c	CTATGTAGTGATGGCGCTTG	Signature-tag specific primer
S004	CGGCCGCCAGTGTGATGG	Signature-tag specific primer
S005	AGATATCCATCACACTGGCG	Signature-tag specific primer
S010b	GCGATGGATTTCTCTGTGTG	Signature-tag specific primer
S014b	CGCTGTCGTTCTTGGGATG	Signature-tag specific primer
S015b	GTGGTTAGTAGGTGGCGAG	Signature-tag specific primer
S023b	TTGCGTGCGGTCGCGATTG	Signature-tag specific primer
S024b	ATGTGTTTCGTTTTAGTGTTCTG	Signature-tag specific primer
S025	GTCCTTTCGGTGGTTATATTG	Signature-tag specific primer
S035	AGTGCGCTTGCGTTTTTTATC	Signature-tag specific primer
S040c	TGGGGGTTGGTGTTGATCG	Signature-tag specific primer
S041c	CGAGCGAGGTATATGTTGAGA	Signature-tag specific primer
S043b	TCTTGGGGTCGCTTGTTCTC	Signature-tag specific primer
S044	GTTCGAGCGCGCACTAAAAAT	Signature-tag specific primer
S045	TATCGAGCAAGCTAGCGAAAG	Signature-tag specific primer
S047d[a]	GGTGATCTTGATGTGGCTATC	Signature-tag specific primer
S048	TAGACGTCATTTTGTCGCTAG	Signature-tag specific primer
S049	GCAAAAACCAACGCAACCGC	Signature-tag specific primer
S060	CTGATGCGTGCTAGGTGGA	Signature-tag specific primer
S061	GTCAGGGGGGGGTGTTTAA	Signature-tag specific primer
S062	TGCGGGGGATCGAGATGTT	Signature-tag specific primer
S063	ACGTCTTTGCGCGTTTTCGA	Signature-tag specific primer
S065b	TTTCGCGGTGGTTAGGGAGT	Signature-tag specific primer
S068	CTGGTTCTAGTGGTTGCTCT	Signature-tag specific primer
S069c	CGATCTCGCGATATGTATTTC	Signature-tag specific primer
S070c	TCGTGTTGGTGGGCTGTATC	Signature-tag specific primer
S074	CAGGGTGGTAGTGGGTTAG	Signature-tag specific primer
S075	CGTCGTTGTTGGTTGTGTCT	Signature-tag specific primer
S076b	ATGTGTGTTGTCATTTTTAGGTC	Signature-tag specific primer
S079b	GTCTATGTGGAGGTATATAGG	Signature-tag specific primer
S083	ACGTCCTTGGTTGTTTTCTCT	Signature-tag specific primer
S084	GAGTGGTGTGCTGGAGTGC	Signature-tag specific primer
S085	TGGGGTCTATTGTTTGAGTTG	Signature-tag specific primer
S086	CTGCGCTAGGTAGATGTTTTT	Signature-tag specific primer
S087	CGTCTTCGAGTGGTCTGTTG	Signature-tag specific primer
S088	TTATGTGGAGTTAGAGTGGGT	Signature-tag specific primer
Srev01	GGACAGGTCGGTCTTGACAA	Generic transposon primer

Primers	Sequence (from 5' to 3')	Purpose
For PCR checks		
p5595d	CTGGATCAGATCTATATTCCG	Test of *virB9* (upstream primer)
LHS059	ATCGTTAATATTTTTTTCATCCAC	Test of *virB9* (downstream primer)
LHS060	GTAAAGTGAACATAGGAAACAG	Test of *virB10* (upstream primer)
pRS16	CTCTCAAAAAAGTACTGATAGG	Test of *virB10* (downstream primer)
LHS061	TTTTACAAGAATTCTGCGATACC	Test of *virB11* (upstream primer)
pRS13	ATCATTGCTTTAAAAGAGCCT	Test of *virB11* (downstream primer)
pRS83	TATTCTTATCTACGCCTTTGCAAA	Test of *virD4* (upstream primer)
pRS157	CTAGTTTTTCTTTTGTGGGGC	Test of *virD4* (downstream primer)
VirB16	TTGGCGGTGACTAAGACAA	Test of *bepA* (upstream primer)
pRS111	CAAAGGCGTAGATAAGAATAAC	Test of *bepA* (downstream primer)
LHS042	TTAAGGAGAGTTTATATGTTAGAG	Test of *bepC* (upstream primer)
LHS043	TAAAAGTTGATACGTTTTTAGCC	Test of *bepC* (downstream primer)
prFS03	GTGAAAAAAAGTCACCCACAAC	Test of *bepD1* (upstream primer)
prFS04	TTACATGGCATAAGACATTCCT	Test of *bepD1* (downstream primer)
prFS01	AAAAAACATCATCCACACCCA	Test of *bepD2* (upstream primer)
prFS02	TTACATGGCATAAGCCATTCC	Test of *bepD2* (downstream primer)
LHS045	AGGAAACATGCATGAAAAAAGAC	Test of *bepE* (upstream primer)
LHS046	GAATTACGTTTGTTTAGCTGGC	Test of *bepE* (downstream primer)
LHS047	GAGCTTGAAAGGAACATACATG	Test of *bepF* (upstream primer)
LHS048	AACGATGATGAGACTATCAGG	Test of *bepF* (downstream primer)
LHS049	ATTAAAAACTTGAAAGGAAATATGC	Test of *bepG* (upstream primer)
LHS050	AATTAAAGCTTATATGAATTTTAGAG	Test of *bepG* (downstream primer)

[a] Not used due to weak PCR product generation

CONCLUDING REMARKS

This PhD thesis had the goal to identify pathogenicity factors of *Bartonella* essential for inducing intraerythrocytic bacteremia during infection of the mammalian reservoir host. The ability to invade erythrocytes of the host is the hallmark of *Bartonella* infection, facilitating the transmission from host to host by blood-feeding arthopods. In the case of human-specific *Bartonella bacilliformis* and *Bartonella quintana*, intraerythrocytic bacteremia leads to clinical manifestations of Oroya fever and trench fever, respectively.Only few other pathogens are also capable of invading mature erythrocytes, like the prokaryote *Anaplasma marginale* (5) or eukaryotic parasites of the phylum Apicomplexa, e.g. *Plasmodium*, the causative agent of malaria. These eukaryotes possess a complicated machinery, the so-called apical complex, to actively invade erythrocytes (for a recent review, see Soldati et al. [11]), which are not capable to phagocytose. How *Bartonella* reaches this intracellular niche is presently not understood. Additionally, bartonellae have first to gain competence for invading erythrocytes in a yet not experimentally proven primary niche, which, however, is considered to include vascular endothelial cells (3). To identify pathogenicity factors *in vivo* that enable the bacteria to colonize its different niches in the host, ultimately resulting in intraerythrocytical bacteremia, I adapted large-scale signature-tagged mutagenesis (STM) to *Bartonella*. STM is a powerful negative selection method allowing to identify those genes of a pathogen that are required for survival in an animal. In the ten years since its first description by David Holden and colleagues (4), 33 bacterial pathogens were investigated 54 published STM studies (see STM review, Table 1). These studies revealed by screening nearly 100000 mutants over 1700 genes involved in pathogenicity. For large-scale *in vivo* screening, traditional random transposon mutagenesis (RTM) is hardly applicable, because each transposon mutant would have to be tested individually in at least one animal. In STM (based on RTM), unique DNA sequences, so-called signature-tags (ST), are introduced into the transposons. Transposon mutants with different STs can then be pooled, reducing substantially the number of animals needed for *in vivo* screening.

For the application of STM in *Bartonella*, I established, in a first step, a RTM system for *Bartonella*, using the *mariner* family transposon *Himar1*. With this, I showed effective RTM in *Bartonella tribocorum* with a frequency of transposition events (transconjugants divided by *B. tribocorum* recipients) of 2.4×10^{-4}. Furthermore, the transposon inserted in a single copy in distinct sites of the chromosome (see STM manuscript, Figure 2). After introduction of 36 STs into the transposon, I generated a mutant library of 3456 tagged mutants (36 x 96). For

detection of the individual STs in the mutant pools, I established a PCR-detection strategy, optimizing the primers for reproducible tag amplification. Using rat-specific *B. tribocorum* in a rat-infection model (9), I screened 3084 STM mutants for an abacteremic phenotype in two rats in parallel after 7 and 14 days of infection, where bacteremia with wild-type *B. tribocorum* peaks. During the screen, Muriel Vayssier-Taussat (Ecole Nationale Vétérinaire Alfort, Maisons-Alfort, France) helped me with the animal experiments and the diploma student Michèle Stöckli with the PCR detection (12). The screen of 3084 ST mutants discovered 359 abacteremic mutant candidates, which I rescreened in newly assembled pools. In this rescreen, the pools were used to inoculate four rats, two like in the primary screen and two including wild-type competition (for details, see STM manuscript, results, "screening of the mutant library"). After these two rounds of screening, a total of 130 abacteremic mutants were shown to be absent from the blood of all infected rats ($n \geq 6$). I mapped the transposon insertion sites on the not yet fully assembled *B. tribocorum* genome (see "Perspective"). Direct sequencing of the transposon flanking sequences on isolated genomic DNA of these mutants was done by Christa Lanz in the lab of Stephan Schuster (Max-Planck Institut für Entwicklungsbiologie, Tübingen, Germany). The transposon insertions in the 130 bacteremic mutants affect 80 genes, which I categorized according to their putative function (see STM manuscript, Figure 3). With these results, I first confirmed already known pathogenicity factors responsible for interaction with the host, like the two type IV secretion systems VirB-D4 (7) and Trw (10). Second, I could also confirm *in vivo* essentiality of so far putative pathogenicity factors, like one of the hemin-binding proteins (HbpB) involved in iron-uptake (6) or the invasion-associated locus protein B (IalB) (2). Third, I identified factors previously unlinked to pathogenesis. These belong to diverse functional classes, predominantly transport or gene-expression regulation, but also cell envelope integrity or metabolism. And forth, I classified a quarter of the identified genes as (conserved) hypothetical. Based on their mutant phenotype in the STM screen, these genes code for novel pathogenicity factors. Of outstanding interest is one large gene, in which I identified five independent transposon insertions and which encodes a *Bartonella*-specific protein (approx. 1501 aa). Apart from a predicted signal peptide, no further obvious domain structure or homology to other proteins available to date indicates a possible function. Its putative surface presentation (or secretion) is supported by the finding that the orthologous regions in *B. henselae* and *B. quintana* code for proteins annotated as surface proteins. In contrast to the typically high sequence conservation and synteny between these three *Bartonella* species, the weak similarity and synteny in this region could reflect host-specific adaptation as it may be speculated for the

region of the *Bartonella* adhesin(s) BadA/VompA-D (see STM manuscript, discussion, "adhesion and invasion"). The fully assembled *B. tribocorum* will allow detailed comparative genomic analyses (see also "Perspective") to understand the common features of *Bartonella* pathogenesis as well as host-specific differences between the *Bartonella* species.

Despite the large number of pathogenicity factors identified, some presumed ones could not be discovered. Possible reasons are: (i) this factor is individually not essential in pathogenesis, (ii) this factor is additionally to its role in pathogenicity essential for *in vitro* survival, (iii) it can be complemented *in trans* intracellularly by paralogous proteins or extracellularly by the orthologous factor of co-infecting wild-type like bacteria, or (iv) the factor is not affected in the STM mutant library due to insufficient mutational saturation. To exclude the last point, I have developed a PCR-screening approach on the entire mutant library. As examplary region to analyze, I chose the *virB/D4/bep* cluster, coding for the VirB/D4 type IV secretion system (7) and its Bep effector proteins (in detail described in *B. henselae* [8]), because it showed a striking imbalanced distribution of abacteremic mutants (see STM manuscript, Figure 4). With the PCR-screening approach, I could show that this imbalance is not due to insufficient mutational saturation in the mutant library, by discovering mutants in *bepA, bepD1, bepD2,* and *bepF*. With growth of these *bep* mutants on complex medium and an infection experiment using single mutants (see STM manuscript, Table 2), I also excluded essentiality for *in vitro* survival and extracellular complementation by Bep orthologs, respectively. Whether these Bep proteins are individually not essential in pathogenesis or intracellularly complemented by paralogous proteins remains to be experimentally proven.

PERSPECTIVE

A large-scale functional genomics approach, like the STM study described here, is only the basis for many follow-up studies, focusing on specific issues discovered during the screen (see STM review, Figure 2 and Table 2, "further characterization"). We started several projects during the course of the STM study to gain more knowlegde about the resulting abacteremic mutants and to design focused experiments to elucidate their specific role in pathogenicity. One is the recently started collaboration with Matthias Christen in the group of Urs Jenal (Biozentrum, Basel, Switzerland), which focuses on a protein without a known function, but containing a GGDEF and an EAL domain, described to regulate the intracellular level of the second messenger cyclic-di-GMP (see STM manuscript, Table 1 and discussion, "unknown function"). Furthermore, I want to briefly introduce three major projects launched due to the STM study, describe my contribution to them, and sketch, how they will complement the STM results and garner deeper insight in the pathogenesis by *Bartonella*. These three projects comprise the *B. triboocum* genome sequencing project, the STM project using a mouse model, and the development and application of *in vitro* assays for *B. tribocorum*.

B. tribocorum Genome Sequencing Project

Knowing the genome sequence of the STM target organism is a prerequisite for mapping the site of mutation and thus the *in silico* characterization of the affected genomic region. Additionally, this enables the analysis of the ST mutants in comparison with genomic data. For example, the STM screen identified at least three different iron-uptake systems, active and individually essential during infection of the mammalian host (see STM manuscript, discussion, "transport"). The open questions, that the genomic sequence of *B. tribocorum* can answer, include: Which components constitute these iron-uptake systems? Are their genes organized in an operon structure? Does the genome contain additional iron-uptake systems? Does it contain conserved known iron-regulatory components, like e.g. *fur* (*feuQ* was also identified in the STM study)?

We want to use the *B. tribocorum* genomic information to answer these kind of questions raised from the STM study. Additionally, we want to use comparative genomics, predominantly with *B. henselae* and *B. quintana* and other α-Proteobacteria, to analyze pathogenicity factors common for *Bartonella* pathogenesis.

For this project, we started in September 2002 a collaboration with the Genomics and Signal Transduction Research Group of Stephan Schuster at the Max-Planck Institut für Entwicklungsbiologie, Tübingen, Germany. I was responsible for all preparational work with *B. tribocorum* (passage through the rat, clonal selection, isolation of genomic DNA for library construction and pulse-field gel electrophoresis) and accompanied the project during all the phases, performed in Basel, and during several coordinative and logistic meetings. In Tübingen, one plasmid library with insert sizes of 2-5 kbp and three Fosmid libraries with insert sizes of 35-43 kbp were used to generate 23435 shotgun and 9377 Fosmid reads. The genome size was estimated to be 2.7 Mbp, so the sequences approx. produced a 7.7-fold genomic coverage and a 72-fold clone coverage. Full automatic assembly by the Phrap software (http://www.phrap.org/phredphrapconsed.html) (and other assembler programs) could not be achieved due to a high degree of repetitive sequences. Günter Raddatz was responsible for all bioinformatics work in Tübingen. Also, the construction of a detailed restriction map of one Fosmid library, done by the diploma student Michèle Stöckli, whom I supervised, together with Christa Lanz in Tübingen, could not solve the assembly problems. With the help of a bacterial artificial chromosome (BAC) library (insert sizes of approx. 120-230 kbp), the master student Philipp Engel, whom I tutor, could assemble the genome correctly with extensive manual curation. Christa Lanz, Günter Raddatz, and Philipp Engel collaborated closely during the gap closure and polishing phase. Presently, the assembly of the *B. tribocorum* genome is finished and can be annotated, which will include a first automated annotation (Günter Raddatz) and the following manual annotation by our lab and the lab of Stephan Schuster (meanwhile at Penn State University, Pennsylvania, USA).

STM in a mouse model

In February 2003, we started a collaboration with Muriel Vayssier-Taussat (see above, "concluding remarks"), who intended to start an STM study using a mouse model of *Bartonella* infection. Since I had successfully established the STM technique in *B. tribocorum*, we invited her to transfer this STM approach to her mouse model. During all the work done here in Basel, I closely collaborated with her. First, we compared the kinetics of mouse infection of two different mouse-specific *Bartonella* species (*B. birtlesii* and *B. taylori*). In parallel, we confirmed, that these species could receive the mutagenesis vector via conjugation, and that the frequency of transopsition events was sufficient for RTM. Subsequently, we constructed a mutant library using *B. birtlesii* as target organism, because of its reproducible *in vivo* kinetics. This mutant library consists of 3456 mutants (96 mutants

each of 36 differently tagged transposons).We also already performed the negative selection screen for the first 576 *B. birtlesii* mutants, which Muriel Vayssier-Taussat analyzed by PCR detection. Preliminary data show 12 attenuated mutants, not detectable in the blood of two mice on day 7, 10, and 14 postinfection. Five of those mutants were also discovered in this STM study. Comparison of the results of both studies will indicate common pathogenicity traits in the genus *Bartonella* and host-specific features among bartonellae infecting different hosts.

***B. tribocorum in vitro* assays**

One major advantage of STM is, that the genes of interest determined in the screen carry already a mutation. So the corresponding mutants can thus directly be used for further phenotypic characterization. To elucidate the specific function of a gene product, one can apply *in vitro* assays, where interaction between the pathogen and its host cell can be closely monitored.

Whereas various aspects of *B. henselae*-endothelial cell interaction can be studied in *in vitro* cell culture assays (for an overview, see Dehio [3]), no *in vitro* system for *B. tribocorum* is described. Michèle Stöckli adapted an assay to study invasion behavior of *B. henselae* into cultured endothelial cells (Ea.hy 926, a fusion of human umbilical vein endothelial cells and lung carcinoma cells) to *B. tribocorum* (12). Testing selected ST mutants, she observed that mutants with a disrupted two-component regulatory system BatSR appear intracellularly in statistically significantly higher number than wild-type bacteria. This could indicate an antiphagocytic effect mediated by BatSR. During these experiments, I contributed to the choice of ST mutants, to the setup of the assays, and to the analysis of the results.

Now, Philipp Engel and I plan to integrate (i) our knowledge of the BatRS regulon of *B. henselae* from transcriptional profiling experiments (1), (ii) the ST mutants with insertions in orthologs of the *B. henselae* BatSR regulon genes to test their phenotype in the invasion assay. With this approach, we want to identify the protein(s) in the regulon of the BatSR system that are directly responsible for the antiphagocytotic phenotype.

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CURRICULUM VITAE

Name:	Luis-Henri Saenz Dürrmeier
Date of birth:	29.09.1974
Place of birth:	Ingolstadt, Germany
Nationality:	German
Family status:	Single

Address	**Office:**	**Private:**
	Biozentrum University of Basel Division of Molecular Microbiology Klingelbergstrasse 50/70 CH-4056 Basel Switzerland Phone: +41-61-267 2139 Fax: +41-61-267 2118 Email: henri.saenz@unibas.ch	Burgfelderstrasse 25 CH-4055 Switzerland Phone: +41-61-321 4884

Research experience:

Mar. 2001 – Apr. 2005	Ph.D. work in the lab of Prof. Dr. Christoph Dehio at the Biozentrum of the University of Basel, Switzerland; title: Signature-tagged mutagenesis in *Bartonella* PhD committee members: Prof. Christoph Dehio; Prof. Urs Jenal; Prof. Guy Cornelis
May 2000 - Dec. 2000	Diploma thesis in the lab of Prof. Dr. Friedrich Götz at the Eberhard-Karls-Universität, Tübingen, Germany; title: Biosynthesis of the pheromone peptide and influence of the δ-toxin on biofilm formation in staphylococci

Educational background:

Apr. 1999 – Apr. 2000	Final exams in microbiology (main focus), parasitology, and virology
Oct. 1995 – Dec. 2000	Studies of biology at the Eberhard-Karls-Universität, Tübingen, Germany
Jul. 1994	Abitur (university-entrance diploma) at the Wernher-von-Braun Gymnasium, Friedberg, Germany

Teaching experience:

Oct. 2001 – Feb. 2002 and Oct. 2002 – Feb. 2003	Tutorage of first-year students: "Introduction into Biology" at the Biozentrum of the University of Basel, Switzerland
May 2001 / May 2002 and May 2003 / May 2004	Tutorage of third-year students: "Blockcourse in Microbiology" at the Biozentrum of the University of Basel, Switzerland
Sept. 2003 – Sept. 2004 and Oct. 2004 – present	Tutorage of a diploma and a master student at the Biozentrum of the University of Basel, Switzerland

Other activities:

Sep. 1994 – Sep. 1995	Civilian service in tutoring school children at the Annakolleg, Augsburg, Germany
Feb. 1996 – Apr. 1998	Repeated short time employment as student trainee in the Chemical Laboratory of Quality Control, MAN B&W Diesel AG, Augsburg, Germany

Poster presentations:

October 2004	Poster at the (EFB) international conference in Copanello (Catanzaro), Italy: "Functional genomics in *Bartonella*" (Saenz H. L., Stoeckli M. C., Vayssier-Taussat M., Lanz C., Schuster S. C., Dehio C.)
October 2004	Poster at the Biozentrum Symposium in Basel, Switzerland: "Functional genomics in *Bartonella*" (Saenz H. L., Stoeckli M. C., Vayssier-Taussat M., Lanz C., Schuster S. C., Dehio C.)

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