



René Bücker (Autor)

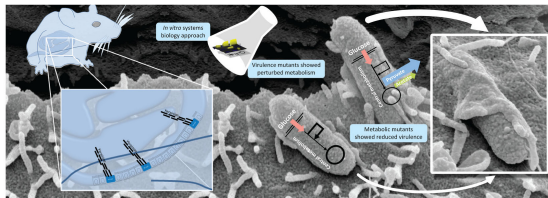
A multi-omics view on the pathogen *Yersinia pseudotuberculosis* – bridging metabolism and virulence



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René Bücker

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1 INTRODUCTION

In 2010, approximately 10 million people died worldwide through infectious diseases, accounting for about 20% of all death cases. More than half of such fatal infections are related to common diseases like diarrhea, lower respiratory infections, or meningitis (Lozano *et al*, 2012). Favorably, intensive research has decreased the danger and death cases of infections. However, in terms of the number of victims, infectious diseases are still a significant burden for public health and further deserve intensive investigation. This is complicated by the emergence of new threats. New pathogens are constantly released from animal reservoirs and from human-adapted agents, respectively, by virulence gene transfer and mutation (Morens & Fauci, 2013). As an example, the emergence of *Yersinia pestis* from the zoonotic bacterium *Yersinia pseudotuberculosis* is responsible for one of the most fatal pandemics in human history resulting in 50 million deaths (Morens *et al*, 2008). Although genetically almost identical, *Y. pseudotuberculosis* and *Y. pestis* differ significantly in terms of host entry and process of infection. *Y. pseudotuberculosis* enters the host via oral uptake and subsequent adhesion to and translocation through intestinal cells (Grützkau *et al*, 1990; Isberg & Leong, 1990) leading finally to diarrhea, enteritis, and colitis (Figure 1). In contrast, *Y. pestis* is transmitted via flea bites (Achtman *et al*, 1999) and causes severe septicemia that mostly leads to death, if it remains untreated (Bosio *et al*, 2012). *Yersinia enterocolitica*, the third pathogenic yersinia member, is phylogenetically more distant to *Y. pseudotuberculosis*, but causes a rather similar infection process (Wren, 2003). At the intersection of two different pathogenic life styles, the analysis of *Y. pseudotuberculosis* provides the opportunity to gain an in-depth understanding of virulence. The infection process of yersiniae is accompanied by constantly changing environmental conditions and the immune

defense mechanisms of the host. *Y. pseudotuberculosis*, e.g., passes the acidic stomach and then enters the different parts of the intestine. Here, *Yersinia* competes with the indigenous microbiota. Every member of this microbial community is perfectly coordinated with one another and inhabits a specific niche (Njoroge *et al*, 2012).

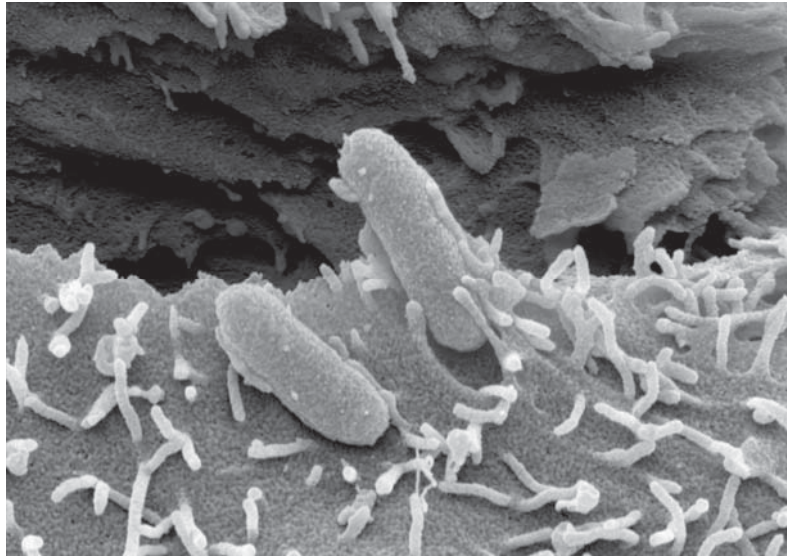


Figure 1. Raster electron micrograph of *Yersinia pseudotuberculosis* on Caco-2 cells (Picture by Manfred Rohde, Department of Molecular mechanisms of streptococci, Helmholtz Centre for Infection Research, Braunschweig, Germany).

Hence, *Y. pseudotuberculosis* faces strong competition for carbon and energy sources and has to defend against the immune system of the host. Thus, a successful colonization relies on accurate adjustment of virulence gene expression to use scarce resources effectively (Njoroge *et al*, 2012). In this context, the question arises, whether virulence factors are controlled by available trigger substances (Lawhon *et al*, 2002) or even by specific metabolic phenotypes as consequence of an integrated output of impact factors. Since systems-wide approaches have been proven useful to find relations between so far unassociated microbial behaviors, their application to pathogenic bacteria promises enhanced understanding of such complex systems.



2 OBJECTIVES

The main object of the present work aims at understanding the role of metabolism in virulence control of the pathogenic model bacterium *Y. pseudotuberculosis*. In anticipation of the complexity of the involved metabolic and regulatory networks, a systems biology approach using state-of-the-art ^{13}C -based fluxome and array-based transcriptome analysis should be developed and applied. As such analyses require high reproducibility and accuracy, a convenient cultivation strategy for *Y. pseudotuberculosis* should be developed first. The established cultivation set-up should subsequently be used to analyze defined gene deletion mutants lacking major virulence regulators with different hierarchical positions within the virulence control cascade. The determined metabolic phenotype, i.e., the fluxome, should then be integrated with transcriptome data to correlate metabolic states with virulence control. The integration should provide new insights into the life style of *Y. pseudotuberculosis*. In addition, the contribution of the core metabolism of *Y. pseudotuberculosis* should be investigated in context of its defense role against external threats like antimicrobial agents. At best, promising novel targets, identified from the systems biology analysis and related to virulence control, should be characterized through profiling of second generation mutants, including mouse infection studies to investigate their relevance *in vivo*. Finally, selective studies should also explore the dynamic of metabolic and regulatory networks of *Yersinia pseudotuberculosis*.



3 THEORETICAL BACKGROUND

3.1 Clinical relevance of *Yersinia pseudotuberculosis*

In mammals, including humans, *Yersinia pseudotuberculosis* causes gut-associated diseases, such as diarrhea, enteritis, and colitis. The threat of *Yersinia pseudotuberculosis* is underdetermined by the fact that it causes severe bloodstream infections with a death probability up to 75% (Mandell *et al*, 2009; Kaasch *et al*, 2012). Several recent foodborne outbreaks in industrial countries like Finland or Japan further indicate an on-going impact of this Gram-negative pathogen on society (Nakano *et al*, 1989; Hannu *et al*, 2003; Jalava *et al*, 2006; Rimhanen-Finne *et al*, 2009). Obviously, *Yersinia* remains a serious threat for foodborne infections. Due to its wide-spread distribution among sheep, deer, pig, hare, and even birds, contamination of vegetable fields by an animal reservoir seems likely (Tauxe, 2004). In addition to its clinical relevance, *Y. pseudotuberculosis* is important as an evolutionary ancestor of *Y. pestis*, the agent of plague (Achtman *et al*, 1999). *Y. pestis* evolved just 1,500 – 20,000 years ago and shows an almost identical genetic background. There seems to be only one exclusive determinant that significantly enhances infection by *Y. pestis* as compared to *Y. pseudotuberculosis*: a plasmid encoded phospholipase D homolog (Achtman *et al*, 1999). Acquisition of plasmid pFra, coding for the phospholipase D homolog, appears sufficient for *Y. pseudotuberculosis* to evolve to one of the world's worst pathogen. Due to this, the investigation of the life style and the infection process of *Y. pseudotuberculosis*, as aimed in this work, is important towards understanding of two human pathogenic bacteria.



3.2 *Yersinia pseudotuberculosis* - Life style and infection process

The typical infection route of *Y. pseudotuberculosis* occurs via oral uptake and binding to the intestinal epithelium (Isberg & Leong, 1990). Contact and invasion of the epithelia is mediated by invasins. It recognizes β_1 chain integrins that are exclusively provided by microfold cells (M cells). After translocation, *Yersinia* attaches to phagocytic cells located within the encountered region between epithelium and Peyer's patches, the subepithelial dome. The immune response is then paralyzed by several *Yersinia* outer proteins (Yop) that are channeled through a type III secretion apparatus into immune cells (Isberg & Barnes, 2001). Subsequently, the pathogen spreads into the lymphatic system, where it rapidly multiplies and colonizes deeper tissues, such as mesenteric lymph nodes, liver, and spleen (Figure 2) (Dube, 2009).

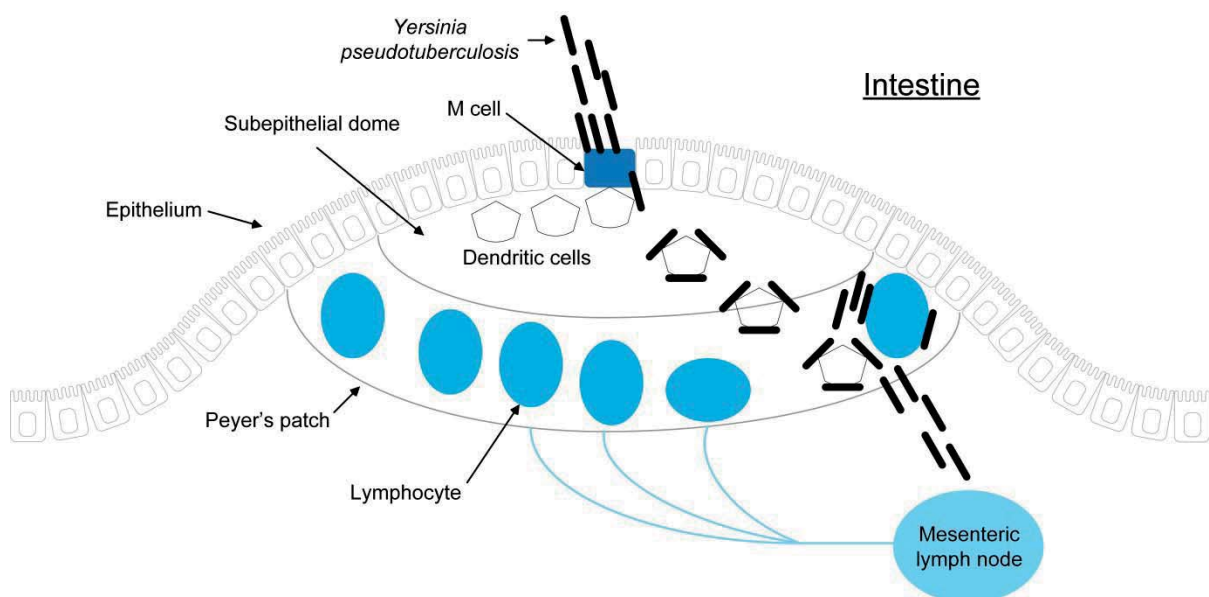


Figure 2. Process of infection. After attachment, internalization, and translocation of microfold cells (M cells), *Yersinia* reaches the subepithelial dome. Here, attachment to dendritic cells and inactivation of the immune defense occurs. Paralyzed dendritic cells then carry the pathogen into the Peyer's patches, where it rapidly multiplies and disseminates into deeper tissues. The figure is adapted from (Mowat, 2003) and (Isberg & Barnes, 2001).

In order to promote its pathogenic life style, *Y. pseudotuberculosis* has developed global regulatory cascades that coordinate physiological processes and virulence



factors and initiate infection by expression of the major invasive factor: invasin. Several regulators of these cascades suggest a link between virulence and core metabolism of the pathogen. Over the last few years, it has become evident that the catabolite repressor protein (Crp) and the carbon storage regulator A (CsrA) are involved in virulence management in addition to their well-known function as metabolic regulators (Figure 3) (Heroven *et al*, 2012a; Heroven *et al*, 2012b). CsrA is a RNA-binding regulator protein, which itself is controlled by the small non-coding RNAs CsrB and CsrC. CsrA is also involved in controlling the major transcriptional regulator of virulence (RovA) and its transcriptional repressor (RovM). In the absence of CsrA, expression of RovM is reduced, and lower levels of the repressor then lead to higher amounts of RovA (Heroven *et al*, 2008) and subsequent expression of invasin, thus mediating attachment to and entry into the intestinal epithelium (Figure 2) (Nagel *et al*, 2001). The Crp protein influences the expression of *rovA* through counter-regulation of the Csr RNAs either directly or through the UvrY response regulator (Figure 3). Crp and CsrA are pivotal for a successful *Yersinia* infection (Heroven *et al*, 2012b). Crp also affects the virulence and metabolism of *Y. pestis* (Zhan *et al*, 2008). In addition to the coordinated expression of virulence factors, the ability to efficiently compete for nutrients is crucial for a successful infection, i.e., the bacteria must outcompete the gut microbiota and persist long-term within the intestinal tract (Chang *et al*, 2004; Hofreuter *et al*, 2008). As described above, the expression of RovA is crucial for the scheduled provision of invasin. In addition to concerted regulation of RovA by Crp, CsrA, and RovM (Figure 3), RovA abundance varies with temperature, osmolarity, pH, growth phase, and nutrient status. Maximum levels are found at ambient temperature (20 - 28°C) and in stationary growth phase.

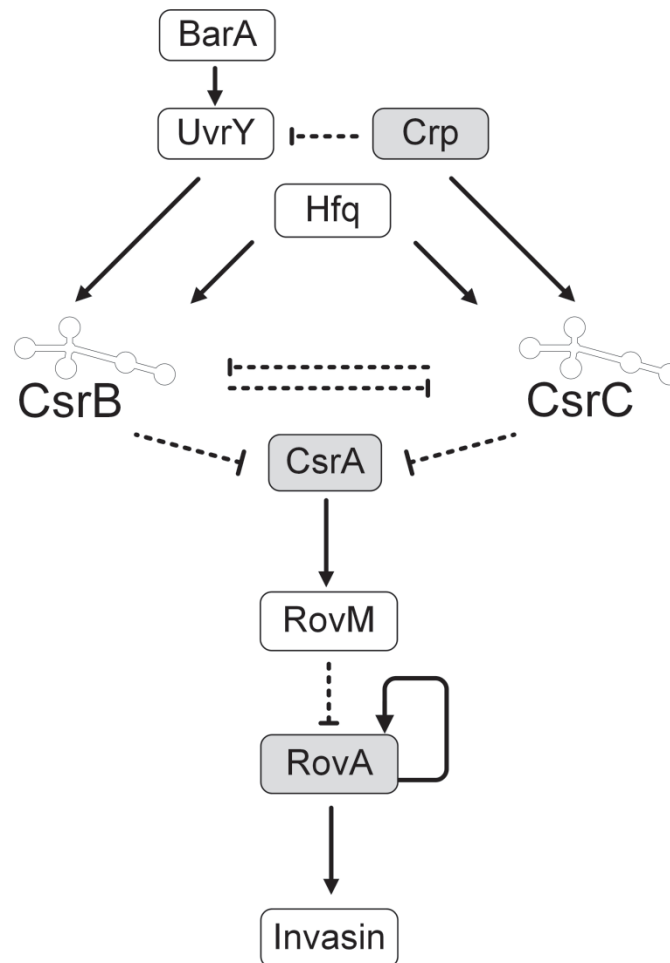


Figure 3. Regulatory network of the virulence genes of *Y. pseudotuberculosis*. The interactions involve activation (arrows) and repression (dashed lines). The impact of the catabolite repressor protein (Crp), the carbon storage regulator protein A (CsrA), and the regulator of virulence A (RovA) on central metabolism was investigated in this work. The corresponding regulators are displayed in grey boxes. This figure is adapted from (Heroven *et al*, 2012b).

During batch culture, *rovA* expression emerges within the mid-log phase and then increases continuously (Nagel *et al*, 2001). An osmolarity level equal to the physiological value of the gut (Sleisenger, 1981) is favorable for *rovA* expression. In contrast, pH lower than seven results in drastic decrease of RovA. Host temperature significantly reduces RovA abundance, independent of further parameters (Herbst *et al*, 2009). Nutrient rich media favor *rovA* expression, whereas minimal media stimulate *rovA* expression only weakly. Additionally, *rovA* expression is auto-regulated (Figure 4) (Nagel *et al*, 2001).

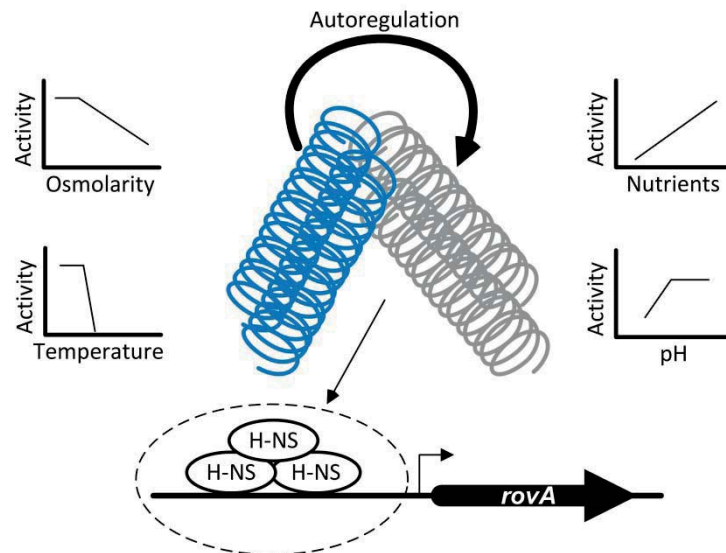


Figure 4. Factors that influence the expression of RovA (regulator of virulence A) in *Yersinia pseudotuberculosis*. Expression is reduced at high osmolarity, low pH values, low nutrient availability (Nagel *et al*, 2001), and at 37°C (Herbst *et al*, 2009). The nucleoid-associated protein (H-NS) binds with high affinity to the promoter region of *rovA* and, thus, inhibits positive autoregulation (Heroven *et al*, 2004). At ambient temperature, RovA shows higher affinity and is able to suppress H-NS. Given plots schematically illustrate the tendency of RovA activity as function of the individual parameters.

Taken together, temperature has a prominent impact on virulence control of *Yersinia pseudotuberculosis*. Therefore, further studies have investigated temperature dependence of RovA in more detail. At host temperature, a reversible conformational change occurs, which leads to reduced promoter affinity to target genes and increased susceptibility to degradation (Herbst *et al*, 2009). Furthermore, the nucleoid-associated protein H-NS binds with high affinity to the promoter of *rovA*, and hence, prevents transcription. At 25°C, a drastically increased promoter affinity of RovA suppresses H-NS binding and leads to readmission of *rovA* expression (Figure 4) (Heroven *et al*, 2004). To date, the temperature dependence of RovA was mainly described qualitatively. A more precise quantitative correlation of RovA levels with temperature would allow the construction of kinetic models towards better understanding of the underlying interaction.



3.3 Virulence promoting metabolism of *Yersinia pseudotuberculosis*

One of the key questions that arise from our current knowledge of *Yersinia's* virulence revolves around the metabolic core machinery of the cell. A first cross-view on *Yersinia's* metabolic repertoire can be obtained from its genome annotation (Kyoto Encyclopedia of Genes and Genomes) (Figure 5). *Y. pseudotuberculosis* harbors phosphotransferase systems (PTS) for uptake of carbohydrates, such as glucose. Glycolytic conversion of glucose 6-phosphate occurs via the Embden-Meyerhof-Parnas (EMP) pathway and the Entner-Doudoroff (ED) pathway, respectively. However, the major role of the ED pathway seems to be related to catabolism of specific carbohydrates, like gluconate (Conway, 1992). In accordance with its close relative *Escherichia coli*, a complete pentose phosphate (PP) pathway for NADPH supply by glucose 6-phosphate dehydrogenase and by 6-phosphogluconate dehydrogenase, respectively, is present in *Y. pseudotuberculosis*. The pyruvate metabolism encompasses anaplerotic PEP (phosphoenolpyruvate) carboxylase, gluconeogenic PEP carboxykinase, and NADP-dependent malic enzyme. For maintaining appropriate NAD/NADH ratios at substrate overflow, *Yersinia* possesses lactate and acetaldehyde/alcohol dehydrogenases. A quinone-reducing pyruvate dehydrogenase delivers quinol as alternative reducing equivalent. Acetyl-CoA can either be metabolized via the tricarboxylic acid (TCA) cycle or via the glyoxylate shunt (Figure 5). Although metabolic regulators are directly involved in the control of crucial infection determinants, metabolic requirements of *Yersinia* for adapting to and surviving in different host niches are largely unknown.

