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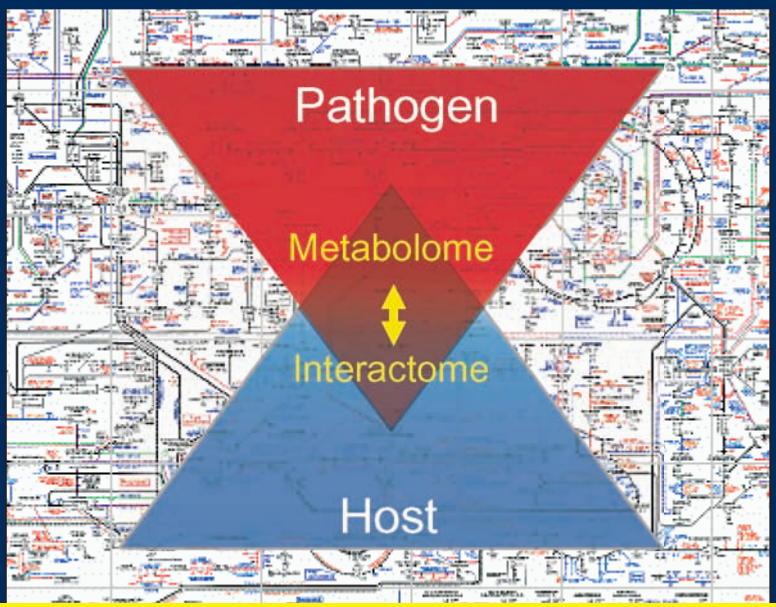
Metabolism Meets Virulence

International Symposium on Metabolism and Bacterial Pathogenesis

Jürgen Heesemann and Michael Hensel (Eds.)



Deutsche Akademie der Naturforscher Leopoldina –
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Metabolism Meets Virulence

International Symposium on Metabolism and Bacterial Pathogenesis

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Editors:

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With 23 Figures and 5 Tables

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Biofilms and Bacterial Communication

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Introduction

The concept of bacterial pathogenicity is changing with the continual increase of knowledge about host-pathogen interaction and co-evolution. Starting with Henle-Koch's postulates to define pathogenic bacteria and the discovery of bacterial exotoxins such as diphtheria toxin, the concept of virulence factors was born. With the beginning of gene cloning and designed gene disruption about thirty years ago, the new subject of Molecular Infection Biology disseminated into natural science and medical faculties. The basic toxin-based concept of microbial pathogenicity was replaced by the dynamic co-evolutionary concept including pathogenicity islands and complex machineries (protein secretion systems) involved in the injection of large sets of anti-host effector proteins into host cells to reprogram the host defense in favor of the pathogens. But the increase of pathogenicity factors from classical exotoxins to diverse modulins, adhesins etc. will not be the entire story of bacterial pathogenicity. Free-living bacteria multiply and survive within a completely different environment in comparison to host-adapted bacteria. Colonization of, or translocation across, host epithelial interfaces by microbes also requires adaptation to the hostile environment caused by host defense and, in particular, by the metabolism of the host. Examples are 'fitness factors' such as iron uptake systems to overcome iron starvation or the reduction or extension of bacterial metabolic pathways. Bacterial genome sequencing, transcriptomic and proteomic approaches, as well as the recently developed highly sensitive bioanalytical techniques now allow in-depth studies of the adaptation of the metabolism of pathogens in the host.

Competitive research in this new field requires a close cooperation of experts of diverse disciplines. This has been put into action by the new priority program SPP 1316 "Host-adapted Metabolism of Bacterial Pathogenicity" of the German Research Foundation (DFG) in September 2008.

The participants of SPP 1316 will investigate how bacterial pathogens adapt their metabolism during colonization of host organisms, how the metabolism of pathogenic bacteria and host organism is interconnected, and which mechanisms of control are active.

The projects of SPP 1316 aim to identify metabolic pathways that are important for the bacteria during infection and to determine the metabolic fluxes. The metabolic reactions of the host organisms and the genetic mechanisms of metabolic adaptation will be discovered. The specific aims of the program are:

– to generate integrated knowledge of the host-adapted metabolism during host pathogen-interactions;

– to generate a network of closely collaborating researchers in infection biology, medicine, microbial physiology, bioanalytics and bioinformatics;
– to develop and optimize approaches of bioanalytics and bioinformatics for the analyses of metabolic pathways during infection;
– to identify novel targets for the development of future antimicrobial strategies based on the analysis of the host-adapted bacterial metabolism.

The high demand for specialized methods and instrumentation has previously hampered studies in this area. Due to a close interdisciplinary collaboration of researchers with complementary expertise in infection biology, bioanalytics and bioinformatics, an efficient analysis of the complex interplay of the metabolism of pathogenic bacteria and their host will now be possible. Moreover, the research groups of SPP 1316 would substantially benefit from an international symposium comprising the topics of the SPP 1316 research program. This was achieved by the symposium "Metabolism meets Virulence". International leading scientists in the field have been invited to represent the 'state of the art' in the following areas:

– General aspects of bacterial metabolism,
– RNA biology in bacterial metabolism and virulence,
– Global regulation of bacterial metabolism,
– Metabolic adaptations of pathogens with extracellular or intracellular life style,
– Biofilms and bacterial communication,
– From parasitic to endosymbiotic lifestyles.

In addition to these topic lectures, the 19 principal investigators of the research projects of SPP 1316 presented their research results as posters. After 4 days of lively and spirited discussions there was no doubt that this symposium gave a great impetus to this new field of research of infection biology.

In this special issue the invited speakers represent their results and perspectives as extended abstracts.

We are grateful for financial support of the symposium by

– German Research Foundation (DFG),
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Keynote Lecture

Nova Acta Leopoldina NF *111*, Nr. 378, 11–16 (2010)

Growth Meets Virulence: Confluence of Two Paths of Microbiology

Frederick C. Neidhardt (Ann Arbor, MI, USA)

With 2 Tables

Abstract

Microbiologists have always been fascinated by two distinct aspects of microbial life: the growth of microbes, and their diverse activities. Microbes have emerged from 3 to 4 billion years of evolution as champions of self-reproduction, the defining property of life; furthermore, microbes are uniquely amenable to biochemical and genetic analysis of growth. Hence microbial growth has attracted the attention of many biologists interested in this central aspect of life. But microbes have also developed during their evolution an astounding array of functional capabilities which are largely independent of growth. For the century-and-a-half that microbiology has been a discipline within biology, microbiologists have generally fallen into two camps based on these two different interests. Today these two threads of microbiology cannot be studied separate from each other, as the theme of this symposium illustrates.

Zusammenfassung

Mikrobiologen waren immer begeistert von zwei unterschiedlichen Aspekten des mikrobiellen Lebens: vom Wachstum bzw. von der Vermehrung der Mikroorganismen sowie ihren unterschiedlichen Aktivitäten und Lebensweisen. Bakterien sind vor etwa drei bis vier Milliarden Jahren als die Meister der Selbstreproduktion entstanden und haben damit die wesentliche Eigenschaft von Leben definiert und auch den Zugang zur genetischen und biochemischen Analyse von Wachstum/Vermehrung ermöglicht. Dieser zentrale Aspekt des Lebens war schon immer der große Anziehungspunkt für das wissenschaftliche Interesse von Biologen. Mikroorganismen haben in ihrer Evolution auch eine erstaunliche Vielzahl von Fähigkeiten entwickelt, die nichts mit Wachstum zu tun haben. Als sich die Mikrobiologie vor ungefähr 150 Jahren als eigenständige Disziplin aus der Biologie entwickelte, waren die Mikrobiologen in zwei Interessenlager gespalten: mikrobieller Stoffwechsel und mikrobielle Pathogenität. Heute können diese zwei Forschungsgebiete nicht mehr getrennt bearbeitet werden, wie dieses Symposium zeigt.

1. Microbial Growth

Those of us interested in growth have been especially influenced by the ideas that flowed in the last half-century from the Paris and Copenhagen schools of microbiology. To Jacques Monod (1949), "The study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of Microbiology […] (T)he growth of bacterial cultures, despite the immense complexity of the phenomena to which it testifies, generally obeys relatively simple laws […]"

The first part of this statement is certainly outdated in this era in which metagenomics and pangenomics have permitted one to study microbes without cultivating them at all. But the second part has been completely vindicated. A half-century of intensive exploration of bacte-

rial growth has yielded a number of principles, or "laws", of growth that are both "simple" and satisfying in their elegance. MONOD's view has been born out by the results of the subsequent 60 years of research on bacterial growth. Intensive exploration by dozens of researchers has yielded a number of principles of microbial growth (cf. NEIDHARDT et al. 1990). Most of these principles have been learned using *E. coli* or *Salmonella*, but are believed to be reasonably universal, that is, applicable directly to prokaryotic cells (Bacteria and Archaea), and perhaps to others as well (Tab. 1).

Tab. 1 Some Principles of Microbial Growth

– At constant temperature, with surplus nutrient, bacteria synthesize all their constituents at near-constant differential rates and divide at a particular cell size, a state called balanced growth.
– Certain major phenotypic characteristics of cells in balanced growth (their size, and macromolecular composition) are coordinated with the absolute growth rate, almost independent of the chemical nature of the medium.
– Over a wide range of growth rates at a given temperature, the rates of chain elongation of proteins, RNA, and DNA vary little.
– Over a wide range of growth rates at a given temperature, the time between replication termination and cell division varies little.
– When environmental conditions change, reducing or increasing the growth rate, the pattern of macromolecule synthesis responds in a consistent pattern: first RNA, then protein, and finally DNA synthesis, achieves the new rate.

MONOD's prediction that relatively simple laws would be discovered about growth was correct. These growth studies, along with the near complete elucidation of metabolic pathways and the components of the genome, encourage the attractive view that whole-cell modeling with predictive capabilities is possible. In fact, modeling is not just *possible*, it is *necessary* to advance to the next phase of understanding cell growth.

Without system modeling, one cannot know if the current understanding of any one regulatory circuit is complete or lacking. Thus, one must ask not just how something works, but how it works within the complete cellular network.

Currently there are (at least) two new considerations related to the goal of modeling cell growth. One is the choice of the cell to be modeled. Not too long ago an issue for microbial physiologists interested in growth and its mechanisms was: which K-12 strain or B/r strain of *E. coli* should be chosen as the paradigm. This choice has not been made easier by the embarrassing fact that we can no longer be certain what *E. coli* is.

Pangenomic studies astonish us with the fact that the creature we call *E. coli* does not have a repertoire of 4,500 genes with which to live its life, rather it has available perhaps 15,000 counting the cousins with which it can interact genetically in nature (USSERY 2008, unpublished).

Bacterial growth (and stress) studies have in large measure inferred the nature and behavior of an individual cell in the population from measurements of populations (BREMER et al. 1996, NEIDHARDT et al. 1996). This average cell is a useful (and necessary) construct, but it is a fiction. It is a virtual cell, and within a population there may be no single cell that fits the calculated dimensions and composition of the average cell. This fact takes on special significance when one realizes that clonal populations of cells show phenotypic heterogeneity under homogeneous and invariant conditions.

Initially, identical cells in a population can become significantly different as a result of bistability (= a dynamic system resting in either of two stable states) brought about by such factors as noise, positive feedback, and hysteresis operating on cellular regulatory networks (Veening et al. 2008). Noise (stochastic variation) is significant because the number of molecules of critical proteins involved in transcription and translation is small. This is especially true of transcription factors when not activated or induced. Noise amplified by positive feedback can result in bistability. A striking example of bistability is the clinically relevant phenomenon called persistence. Persistence (Bigger 1944, Moyed and Broderich 1983, Veening et al. 2008) is a striking example of bet-hedging. Persister cells are those few cells in a population that have the ability to survive antibiotic treatment. Persister cells, it is now understood, are impeded in growth, and though inherently sensitive to an antibiotic, are protected by their slow or non-growth. Persister cells arise from normal cells by stochastic processes that are epigenetic.

Summary: Studies focused on modeling the growth of individual cells must acknowledge that the basis of much of the data about growth is derived from population measurements under laboratory conditions, not individual cells in a changing environment.

2. Microbial Activities

Most bacteriologists are concerned with microbial processes and activities that are independent of growth. The variety of activities is staggering. Table 2 lists merely those that come to mind from current studies.

Tab. 2 Some non-growth activities of bacteria

Motility/taxes	Biofilm formation	Quorum sensing
Secretion	Export	Survival
Adhesion	Sporulation	Commensalism
Virulence	Community	Stress responses
Resistance	Persistence	Germination
Stationary phase	Luminescence	Colonization

Given the significance of these varied manifestations of microbial effects on the environment and on human beings, the intensity with which molecular microbiologists have focused on growth would seem to be disproportionate to its importance in microbe/human interaction. Some critics have maintained that exponential growth is an artifact of the laboratory and is largely irrelevant in the real world. Even the fact that bacteria respond to stochastic changes in gene expression caused by environmental changes through bistable switches may make most growth studies misleading, if not irrelevant.

This view that growth physiology is not central to understanding virulence demands examination.

Among the largest regulatory networks in the bacterial cell are carbon catabolite repression and the stringent response. Even a cursory perusal of the current research into molecular pathogenesis reveals the central role of these two systems.

2.1 Carbon Catabolite Repression

Carbon catabolite repression refers to the growth-related regulation by bacteria of their utilization of carbon sources when presented with an environment that offers a choice. Well studied in *Escherichia coli*, this regulatory behavior is widespread in the microbial world (reviewed in Görke and Stülke 2008). In the firmicutes (Gram-positives with low G-C DNA content) work on *Bacillus subtilis* has shown that a global regulatory protein, CcpA, contributes to catabolite repression by inhibiting the induction of catabolic operons for other carbon sources when glucose is present in the environment. This behavior helps result in the economy of preserving secondary substrates for later use rather than squandering them needlessly while the favored glucose is available. The regulation is achieved, in part, by the ability of Ccp, when complexed with a phosphorylated form of the protein HPr, to act as a transcriptional repressor. Phosphorylation of HPr from fructose-1,6-bisposphate and glucose-6-phosphate is a key signal to the cell that glucose is being utilized by the cell (reviewed in Görke and Stülke 2008).

In the human pathogen, *Streptococcus pyogenes*, the analogous CcpA protein is central to virulence. In addition to activating the expression of several genes important to virulence, CcpA directly activates the gene producing the protein Mga, a master regulator of virulence in this organism. Mga governs genes for adhesion, internalization, and immune evasion. Likewise, in both *Staphylococcus aureus* and *Streptococcus gondii*, CcpA has been shown to contribute to antibiotic resistance (by, as yet, undiscovered mechanisms). In enteric bacteria, one gene involved in catabolite repression (*crp*) is essential for the expression of virulence genes (reviewed in Görke and Stülke 2008).

2.2 The Stringent Response and Magic Spot

When growing bacterial cells become restricted for either a required amino acid or for their carbon and energy source, they synthesize the nucleotides pppGpp (guanosine 5'triphosphate, 3'diphosphate) and ppGpp (guanosine 5'diphosphate, 3'diphosphate) from ATP and GTP. In enteric bacteria these "magic spot" nucleotides, collectively termed (p)ppGpp, are made on ribosome-bound RelA protein in the case of amino acid restriction, and from the cytosolic protein SpoT in the case of stress and energy starvation in *E. coli*. Magic spot accumulation is rapid, and likewise, its effects are also rapid; binding to RNA polymerase, these nucleotides alter the transcriptional pattern of the cell to a massive extent, activating genes for fatty acid oxidation, glycogen synthesis, nucleotide catabolism, amino acid synthesis and cell division machinery, while diminishing transcription of genes for DNA replication, ribosome synthesis, nucleotide biosynthesis, and phospholipid biosynthesis. Indirectly, by activating the general stress response, central metabolism is accelerated and cell morphology is altered. Moreover, there is evidence that during balanced growth the activity of ppGpp is the major controlling factor in matching ribosome synthesis to the cellular growth rate. A recent review summarizes the current understanding of this important system (Potrykus and Cashel 2008).

Clearly this is a gigantic regulatory network, and its elucidation and function have occupied bacterial growth physiologists for almost half a century. Current studies on molecular pathogenesis are revealing that the operation of this network is crucial for the virulence of many pathogens. The list currently includes *Salmonella typhimurium*, *Legionella pneumophila*, *Escherichia coli* (EHEC), *Pseudomonas aeruginosa, Campylobacter jejuni*, *Brucella*

abortus, *Listeria monocytogenes*, *Borrelia burgdorferi*, *Vibrio cholera*, and *Mycobacterium tuberculosis* (POTRYKUS and CASHEL 2008).

A particularly well studied example is that of *L. pneumophila*, a ubiquitous bacterium residing largely in biofilms or within freshwater protozoa. When inhaled by humans in aerosols of contaminated water, *L. pneumophila* can invade alveolar macrophages, producing a potentially fatal pneumonia. This bacterium alternates between a replicative and a transmissive state, both in broth and in its host. The two phenotypes differ in many ways, including the cell's rates of protein synthesis and DNA replication, stress resistance, ability to evade lysosomes, flagella-mediated motility, Na^+ sensitivity, contact cytotoxicity, beta-hydroxybutyrate storage granules, and the ability to recruit the endoplasmic reticulum (see reviews: MOLOFSKY and SWANSON 2004, HILBI et al. 2007).

Within the host's macrophages, the replicative state results in multiplication of the bacteria until the host cell nutrients are exhausted. At this point the bacteria differentiate into the transmissive state enabling them to survive the dearth of nutrients and transmit themselves into a new macrophage. It turns out that the classical stringent response system that is an essential component of bacterial growth, is also an essential component of the differentiation process. In brief, the current working models indicate that SpoT might monitor the macrophage's metabolic state through an alteration of the bacterium's acyl-carrier protein (ACP), which interacts with SpoT, causing the latter to produce (p)ppGpp. Elevated (p)ppGpp then activates the regulatory cascade responsible for transcriptional regulation of the gene set that produces the differentiation from the replicative into the transmissive state of the bacterium (DALEBROUX et al. 2009, EDWARDS et al. 2009). A central feature of bacterial balanced exponential growth is thus also a key part of virulence.

3. Summary

Studies of bacterial virulence are a productive way to study bacteria in the natural settings that have shaped their evolution. Whether one's interest is focused on growth mechanisms or on processes unrelated to growth, neither aspect can be understood independent from the other. The contribution of this symposium is to highlight the new frontier: the intersection of microbial physiology and microbial virulence, which is a paradigm for future studies that will integrate molecular approaches in the laboratory and in natural environments.

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General Aspects of Bacterial Metabolism

Nova Acta Leopoldina NF *111*, Nr. 378, 19–23 (2010)

Transposon-mediated Directed Mutation

Zhongge Zhang and Milton H. Saier Jr. (La Jolla, CA, USA)

With 2 Figures

Abstract

Directed mutation is a process proposed to allow beneficial mutations to occur at higher frequencies if they relieve the stress conditions that enhance their frequencies. Until now, this process has been controversial, and in no case has such a mechanism been defined. We have identified a novel mechanism involving gene activation by the transposon, IS5, in *Escherichia coli*. *crp* deletion mutants mutate specifically to glycerol utilization (Glp^+) at a rate that is enhanced by glycerol or the loss of the glycerol repressor and depressed by glucose. Of the four tandem GlpR-binding sites (*O1*–*O4*) upstream of *glpFK*, *O4* specifically controls *glpFK* expression while *O1* primarily controls mutation rate in a process mediated by IS5 hopping to a specific site upstream of the *glpFK* promoter. The results establish the principle of directed mutation, define the mechanism involved and identify the proteins responsible.

Zusammenfassung

Unter „gerichteten Mutationen" versteht man das vermehrte Auftreten nützlicher Mutationen, die von einer Mangelsituation ausgelöst werden und diese beheben. Diese Vorstellung wird derzeit sehr kontrovers diskutiert, und es ist kein Mechanismus für das Entstehen dieser Mutationen bekannt. Wir haben einen solchen neuartigen Mechanismus der Genaktivierung durch das Transposon Tn*5* in *Escherichia coli* gefunden. Stämme mit *crp*-Deletionen zeigen eine erhöhte Mutationsrate zu Glycerin-verwertenden Mutanten, wenn Glycerin im Medium vorhanden ist sowie bei Verlust des Glycerin-Repressors oder bei Abwesenheit von Glukose. Der Glycerin-Repressor GlpR besitzt vier benachbarte Bindestellen (*O1*–*O4*) vor dem *glpFK*-Promotor, von denen *O4* für die Regulation von *glpFK* wichtig ist, während *O1* die Häufigkeit kontrolliert, mit der IS*5*-Insertionsmutanten stromaufwärts von *glpFK* auftreten. Diese Ergebnisse beweisen das Konzept der „gerichteten Mutationen" und beschreiben einen Mechanismus ihrer Entstehung sowie die daran beteiligten Proteine.

1. Introduction

It is a basic principle of genetics that the likelihood of a particular mutation occurring is independent of its phenotypic consequences. The concept of directed mutation, defined as genetic change that is specifically induced by the stress conditions that the mutation relieves (Cairns et al. 1988), challenges this principle (Rosenberg 2001, Foster 1999, Wright 2004). The topic of directed mutation is controversial, and its existence, as defined above, has been strongly questioned (Roth et al. 2006).

Part of the justifiable skepticism concerning directed mutation resulted from experiments that appeared to demonstrate this phenomenon, but were subsequently shown to be explainable by classical genetics (Roth et al. 2006). Mutation rates vary with environmental conditions (e.g., growth state) and genetic background (e.g., mutator genes) (Wright 2004, Foster 2005), but this does not render the mutation "directed". To establish the principle of directed

mutation, it is necessary to demonstrate the phenomenon, characterize the mechanism responsible and identify the proteins involved.

One frequently encountered type of mutation results from the hopping of transposable genetic elements, transposons, which can activate or inactivate critical genes (Chandler and Mahillon 2002). For example, activation of the normally cryptic β-glucoside (*bgl*) catabolic operon in *E. coli* can be accomplished by insertion of either IS1 or IS5 upstream of the promoter (Schnetz and Rak 1992).

The *E. coli glp* regulon consists of five operons, two of which (*glpFK* and *glpD*) are required for aerobic growth on glycerol (Lin 1976). Both operons are subject to negative control by the DNA-binding *glp* regulon repressor, GlpR (Zeng et al. 1996), which also binds glycerol-3-phosphate, the inducer of the *glp* regulon. The *glpFK* operon is additionally subject to positive regulation by Crp complexed with cAMP although *glpD* is not (Weissenborn et al. 1992). The *glpFK* regulatory region contains four GlpR-binding sites, *O1–O4*, and two Crp-binding sites which overlap *O2* and *O3* (Fig. 1*A*). The strong Crp dependency of *glpFK* transcription is reflected by the fact that *crp* mutant cells are unable to utilize glycerol.

2. Results

2.1 Glp⁺ Mutations in a crp Genetic Background

When *crp* cells were incubated on solid glycerol minimal medium, Glp⁺ colonies appeared (Fig. 1*B*). We tested the growth of a *crp* Glp⁺ strain on glycerol in defined liquid medium. The growth rate was greater than that of wild type (*wt*) *E. coli* (Zhang and Saier 2009a).

The relative rates of Glp⁺ mutation were determined in minimal and complex media (Fig. 1*B*). On glycerol plates, colonies first appeared after 3 days, although *wt* and *crp* Glp⁺ *E. coli* cells formed visible colonies in < 2 days. New colonies continued to appear at increasing rates thereafter. When the same cells were plated as before, but variable numbers of *crp* Glp⁺ mutant cells were included with the *crp* cells before plating, colonies appeared from the *crp* Glp⁺ cells within two days, and new Glp⁺ mutants arose at the same rate as before (Fig. 1*C*). Thus, the Glp⁺ mutants arising from *crp* cells on these plates were not present in the cell culture initially plated, and no growth inhibitor was present. The rate of mutation on glycerol plates proved to be 10-times higher than in minimal sorbitol or complex LB medium, and it was over 100-times higher than in glucose medium.

Mutation proved to be due to IS5 hopping to a discrete site, 126.5 bp upstream of the transcriptional start site, and always in the same orientation. Only the downstream 177 bp of IS5 was required for activation of the *glpFK* operon, and this proved to be due to the presence of a permanent bend and an overlapping IHF-binding site, each of which was responsible for half of the activation (Zhang and Saier 2009b). This mechanism of activation, presumably involving DNA looping, was also demonstrated for the lactose (*lac*) operon in a *crp* genetic background of *E. coli*.

2.2 Dependency of the Glp⁺ Mutation Rate on GlpR

Glycerol is phosphorylated by GlpK to glycerol-3-phosphate which binds to and releases GlpR from its operators (Lin 1976). When GlpR dissociates from its operators, a conforma-

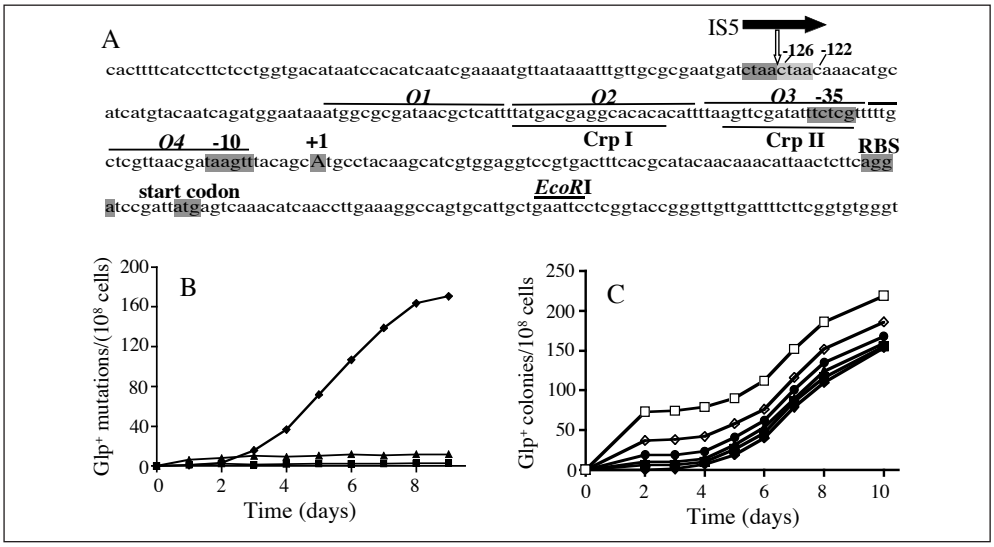

Fig. 1 The appearance of Glp⁺ mutations in a *crp* genetic background. (*A*) The *glpFK* promoter region. The transcriptional initiation site (+1), the –10 and –35 hexamers, the ribosome-binding site (RBS) and the start codon for *glpF* are shaded. The GlpR-binding sites (*O1–O4*; lines above the sequence) and Crp-binding sites (CrpI and CrpII; lines under the sequence) are also shown. The location of the IS5 element upstream of the promoter in *crp* Glp⁺ cells is indicated by the vertical white arrow below the horizontal black arrow representing IS5. The two 4-nucleotide direct repeats (ctaa) caused by IS5 insertion are shaded dark and light gray, respectively. (*B*) Glp⁺ mutations in various media: solid M9 minimal media + 1 % glycerol (♦), 0.01 % glucose (■) or 1 % sorbitol (▲). *crp* cells from an overnight culture (from a single colony) were applied onto agar plates, and the plates were incubated at 30 ºC. On glycerol minimal plates, mutations were determined by the presence of colonies on the plate. On sorbitol and glucose plates, mutations were determined by washing all cells off the plates and measuring colony formation both on LB plates (total cells) and on minimal glycerol plates (Glp⁺ cells) for 36–48 h. (*C*) The same minimal glycerol agar plates, where the *crp* mutant cells were plated together with various numbers of *crp* Glp⁺ cells. Various numbers of *crp* Glp⁺ cells (□, 72; ◊, 38; •, 19; ▲, 10; ■, 5; and ♦, 0) were mixed with *crp* cells and then applied onto M9 glycerol agar plates.

tional change might be transmitted through the DNA, promoting insertion of IS5 at the target CTAA site upstream of the *glpFK* promoter. In other words, GlpR-binding could have two functions: repression of gene expression and suppression of IS5 transposition to the upstream activating site.

To test this possibility, the *glpR* gene was deleted, and the rates of appearance of Glp⁺ mutations in the *crp glpR* double mutant background were measured in the absence and presence of glycerol. The numbers of Glp⁺ cells arising was 10-fold higher in the *crp glpR* double mutant than in the *crp* mutant when glycerol was absent. In the presence of glycerol, the loss of GlpR was without effect. Thus, deletion of *glpR* is equivalent to inclusion of excess glycerol in the growth medium. Overexpression of *glpR* decreased mutation rate to background levels.

2.3 GlpR Operators Differentially Control glpFK Expression and Glp⁺ Mutation Rate

There are four GlpR-binding sites, *O1–O4*, in the upstream *glpFK* operon regulatory region (see Fig. 1*A*) identified by DNA footprinting (Zeng et al. 1996). We mutated the far upstream

site (*O1*) and the far downstream site (*O4*) and compared the effects on *glpFK* expression using a *lacZ* reporter gene fusion construct versus mutation rate to Glp+ during growth in LB medium. Mutation of *O4* increased *glpFK* operon expression about 5-fold, although mutation of *O1* was almost without effect. By contrast, loss of *O1* yielded a 7-fold increase in mutation rate, although loss of *O4* had only a 2-fold effect on mutation rate. We confirmed that IS5 was always in the same position and orientation.

3. Discussion

Directed mutation has been defined as a genetic change that is specifically induced by the stress condition that the mutation relieves (see 1. Introduction), but in no case has such a mechanism been determined. We have demonstrated that mutations in the *glpFK* control region, allowing growth of *E. coli crp* mutants on glycerol, are specifically induced by the presence of glycerol, and that the glycerol regulon repressor, GlpR, which binds to its four operators (*O1–O4*) in front of the *glpFK* operon (Fig. 1*A*) and is displaced from these sites when α-glycerol phosphate binds allosterically (Freedberg and Lin 1973), not only controls gene expression, but also controls mutation rate. Our results establish that *O4*, which overlaps the –10 region, primarily controls gene expression, *O2 and O3*, which overlap the two Crp-binding sites and the –35 region, presumably antagonize activation by the cyclic AMP-Crp complex, and *O1* primarily controls IS5 hopping into the specific CTAA site, 126 base pairs upstream of the *glpFK* transcriptional start site in a reversible process (Zhang and Saier 2009a, b, Zhang et al. 2010). The mechanism may involve DNA change in DNA supercoiling and secondary structure. The results serve to dissociate the two functions of GlpR.

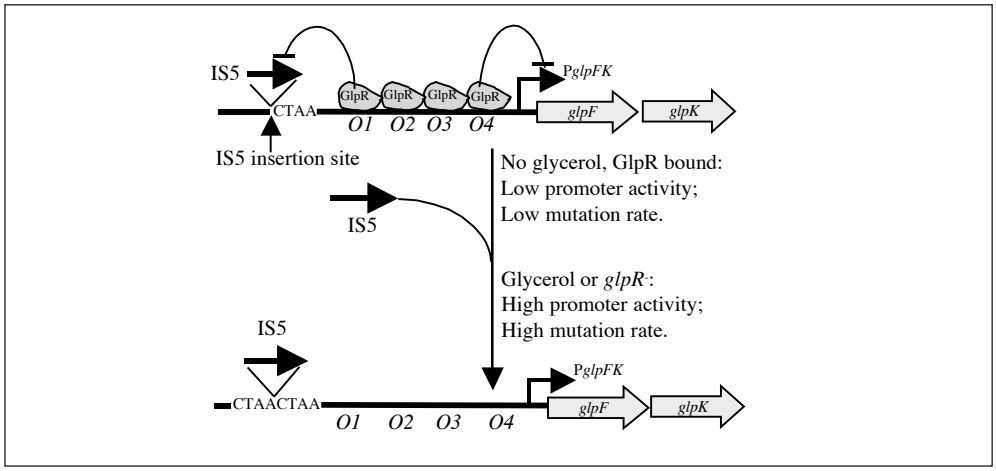

Fig. 2 Schematic diagram illustrating GlpR-mediated control of (right) *glpFK* transcription and (left) the rate of IS5 hopping (directed mutation) into the site upstream of the *glpFK* promoter. With GlpR bound to its operators (*O1–O4*) (in the presence of GlpR and the absence of glycerol), transcription and IS5 hopping both occur at low rates. When GlpR is not bound to its operators (in the absence of GlpR or in the presence of glycerol), both transcriptional initiation and IS5 hopping increase about 10×. Binding of GlpR to operator *O1* blocks IS5 insertion, while binding of GlpR to operator *O4* blocks transcription as indicated.

The mechanism of *glpFK* activation provides relief from starvation and therefore could have been selected during evolution. It appears to be a genuine example of "directed mutation", with mutation occurring at a greater rate under conditions that allow benefit to the organism (Zhang and Saier 2009a). The fact that mutation rate is influenced by the presence of glycerol in a process mediated by the glycerol repressor provides a mechanistic explanation for IS5-mediated directed mutational control. This mechanism, illustrated in Figure 2, may provide a partial explanation for the presence of four GlpR-binding sites in the control region of the *glpFK* operon.

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Climate Change and Infectious Diseases

International Conference

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in Zusammenarbeit mit der Indian National Science Academy, dem Alfried Krupp Wissenschaftskolleg Greifswald und dem Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit

Alfried Krupp Wissenschaftskolleg Greifswald
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Krankheitserreger können sich heute binnen kurzer Zeit weltweit verbreiten und so sowohl gesundheitliche als auch wirtschaftliche Schäden verursachen, wenn sie auf geeignete Umweltbedingungen treffen. Zoonosen, vom Tier auf den Menschen übertragbare Krankheiten, gewinnen dabei zunehmend an Bedeutung. Der Band enthält die Beiträge eines Symposiums, das die neuesten Erkenntnisse zum Klimawandel und zum Einfluss klimatischer Faktoren auf Infektionskrankheiten von Experten der Klimaforschung, der Biologie und der Infektionsmedizin zusammenführte. Erörtert werden auch Einflüsse von klimatischen Veränderungen auf die Evolution und die Biodiversität. Die Bedeutung des Klimawandels für das Auftreten von Infektionskrankheiten wird anerkannt, auch wenn konkrete Auswirkungen bisher nur schwer zu dokumentieren sind. Insbesondere der Überwachung von Vektor- und Reservoirpopulationen sowie der entsprechenden Erreger kommt daher eine große Bedeutung zu, wobei die Untersuchungen langfristig angelegt sein müssen, um Dynamiken in längeren Zeiträumen zu erfassen.

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Carbon Catabolite Regulation in AT-rich Gram-positive Bacteria

Gerald Seidel and Wolfgang Hillen (Nürnberg/Erlangen)

With 4 Figures and 1 Table

Abstract

The mechanisms of carbon catabolite regulation in bacteria are sensitive and complex. The AT-rich Gram-positive bacteria make use of a protein kinase, HPrK/P, to sense the physiological situation and deliver the signals to a master regulator called CcpA. This is also able to itself sense the presence of glycolytic intermediates by direct binding and use this combined information to repress or activate the expression of many different types of genes, including, amongst others, virulence genes. This article summarizes some of the mechanisms involved in signal processing and regulation by CcpA.

Zusammenfassung

Die Mechanismen von Bakterien zur Katabolitregulation sind empfindlich und kompliziert. AT-reiche Gram-positive Bakterien nutzen die Proteinkinase/-phosphorylase HPrK/P, um den physiologischen Zustand der Zelle zu erkennen und die entsprechenden Signale an das übergeordnete Regulatorprotein CcpA zu senden. CcpA selber kann zusätzlich die Anwesenheit glykolytischer Intermediate durch allosterische Bindung wahrnehmen. Es integriert diese Signale in seine Aktivität zur Repression oder Aktivierung der Expression von vielen Genen, u. a. auch von Virulenzgenen. Diese Übersicht fasst einige Mechanismen zur Signalverarbeitung und der daraus resultierenden Genregulation zusammen.

1. Introduction

Carbon catabolite regulation (CCR) refers to the ability of bacteria to specifically select, out of many nutrients present in the environment, the carbon source allowing the fastest growth rate. At the same time, the consumption of carbon sources which would lead to slower growth rates is actively repressed. Since the optimization of growth rates in the presence of various nutrients is a very important evolutionary driving force, the regulons developed by bacteria to accomplish this task are extremely sensitive, and the respective mechanisms are very sophisticated. The mechanism of CCR used by *E. coli* has long been viewed as the paradigma of such regulatory circuits. However, the main components of catabolite regulation in *E. coli*, namely the catabolite activator protein and cAMP, are not present in many other bacteria. The mechanism underlying catabolite regulation in *Bacillus subtilis* and many other AT-rich Gram-positive bacteria (Fig. 1) has been elucidated and is distinct from the one operating in *E. coli* (Deutscher et al. 2006, Görke and Stülke 2008, Stülke and Hillen 1999, 2000).

AT-rich Gram-positive bacteria make use of a protein kinase called HPr kinase/phosphorylase (HPrK/P) to sense the carbon flux through glycolysis by the concentrations of fructose-1,6-bisphosphate (FBP), ATP and inorganic phosphate (Pi). An overview of the regulatory mechanisms is shown in Figure 1. The former two activate the protein kinase activity of HPrK/P while the latter activates its phosphorylase activity. The target protein of HPrK/P is the histidine containing protein HPr which also functions as a phosphotransfer protein in the phosphotransfer sugar uptake system (PTS). That phosphate transfer occurs via phosphorylation of the His15 residue by enzyme I and delivery of this phosphate to the enzyme IIA domain. In contrast, HPrK/P phosphorylates HPr at the Ser46 residue. HPrSerP is a cofactor for the catabolite control protein A (CcpA) which can repress or activate catabolic genes by binding to so-called *cre* (*catabolite responsive element*) sequences (Poncet et al. 2004). Binding of the CcpA-HPrSerP complex to *cre* can lead to either the repression or activation of the affected gene. At σ^A-dependent promoters repression is accomplished by binding downstream of the promoter while activation requires binding to *cre* sequences located upstream of the promoter. Furthermore, glycolytic intermediates such as glucose-6-phosphate (Glc-6-P) and FBP directly enhance the formation of the CcpA-HPrSerP-*cre* complex and, thus, also couple CCR to the carbon flux through glycolysis. CcpA is a global regulator in AT-rich Gram-positive bacteria because the expression levels of 80 to 300 genes are affected when CcpA is inactivated (Blencke et al. 2003, Lulko et al. 2007, Moreno et al. 2001).

A secondary substrate for the HPrK/P protein has been identified which plays no role in PTS. This protein is called Crh (catabolite responsive HPr) and shows sequence similarity to HPr. However, it does not contain the His15 residue required for phosphotransfer in the PTS, but the Ser46 residue at which it is phosphorylated to CrhP by HPrK/P. The contribution of Crh to catabolite regulation in *B. subtilis* is modest, because deletion of the *crh* gene only leads to a phenotype when *ptsH* encoding HPr has also been inactivated. One report claims that Crh is the main component necessary for the regulation of *citZ*, encoding a subunit of citrate synthesis (Warner and Lolkema 2003). Here we summarize the mechanisms by which HPr and Crh contribute to catabolite regulation in *B. subtilis*.

2. Molecular Interdependencies of CcpA-Mediated Carbon Catabolite Regulation

2.1 Qualitative and Quantitative Characteristics of CcpA-mediated Catabolite Regulation in B. subtilis

Surface plasmon resonance measurements (SPR) showed only interactions between CcpA and seryl phosphorylated HPr and Crh, but not with their unphosphorylated forms (Fig. 2*A*). As their reaction kinetics are very fast, they could not be quantified by kinetic analysis. Equilibrium measurements showed weak binding of HPrSerP and CrhP to CcpA with K_D values in the low µM range (see Tab. 1). The equilibrium constant of CcpA-HPrSerP is only about 4-fold larger than that of CcpA-CrhP. In the presence of FBP or Glc-6-P the K_D of CcpA-HPrSerP is about 10-fold lower than that of CcpA-CrhP, because only the interaction of HPrSerP with CcpA is stimulated by these gylcolytic intermediates (Fig. 2*B*). Both, FBP and Glc-6-P, were later shown to bind to the effector binding cleft of CcpA, which is the conserved effector-binding moiety of LacI/GalR family regulators (Schumacher et al. 2007).

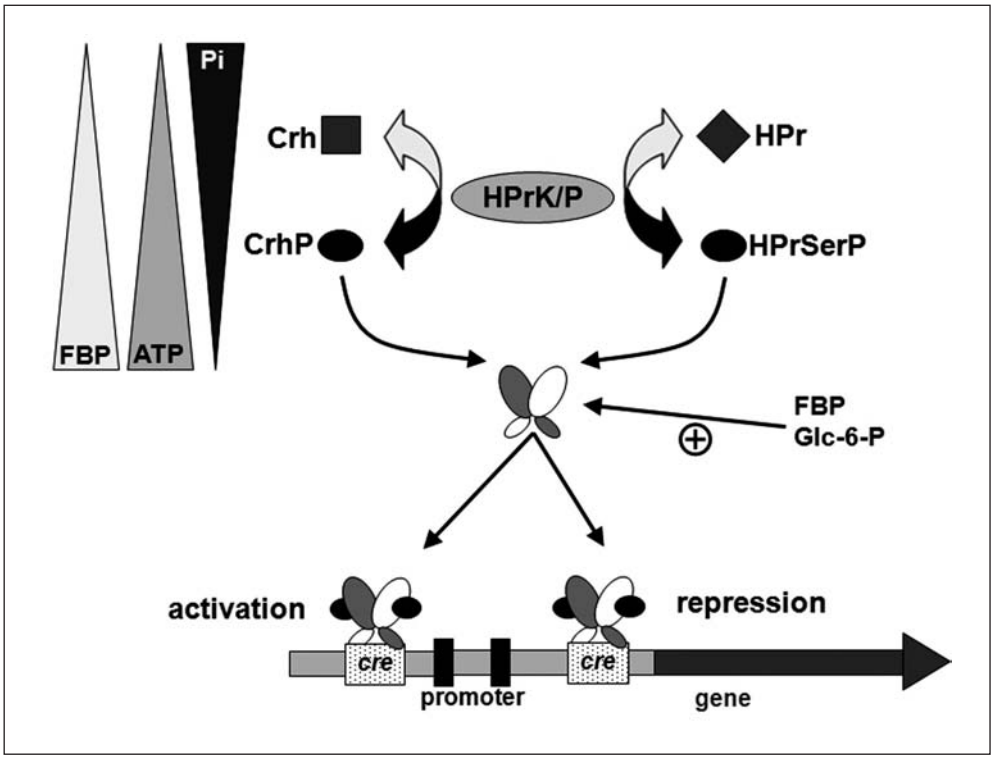

Fig. 1 Carbon catabolite regulation in Gram-positive bacteria. In the presence of HPrSerP or CrhP the catabolite control protein A (CcpA) acts either as a repressor or as an activator of genes by binding to *catabolite response elements* (*cre*) located downstream or upstream, respectively. HPrSerP or CrhP are formed by seryl phosphorylation of HPr or Crh, respectively, mediated by the HPrkinase/phosphorylase (HPrK/P) in the presence of high ATP and fructose-1,6-bisphosphate (FBP) concentrations. *Vice versa*, at low ATP and FBP, but high phosphate (Pi) concentrations, HPrSerP and CrhP are dephosphorylated by HPrK/P by phosphorylation of phosphate yielding pyrophosphate and HPr or Crh. Moreover, FBP and Glc-6-P were shown to stimulate CcpA-*cre* binding.

Tab. 1 Equlibrium constants from HPrSerP-CcpA and CrhP-CcpA complexes, and rate and equilibrium constants from HPrSerP-CcpA-*cre* complexes

Equilibrium and rate constants in the absence of FBP				
Interactant 1	Interactant 2	k_a in $M^{-1}\cdot s^{-1}$	k_d in s^{-1}	K_D in M
HPrSerP	CcpA	n.d.	n.d.	$4.8 \pm 0.4 \times 10^{-6}$
CrhP	CcpA	n.d.	n.d.	$19.6 \pm 2.5 \times 10^{-6}$
HPrSerP-CcpA	*xylcre*	$3 \pm 1 \times 10^{6}$	$2.0 \pm 0{,}4 \times 10^{-3}$	$6 \pm 3 \times 10^{-10}$
Equilibrium and rate constants in the presence of FBP				
Interactant 1	Interactant 2	k_a in $M^{-1}\cdot s^{-1}$	k_d in s^{-1}	K_D in M
HPrSerP	CcpA	n.d.	n.d.	$1.7 \pm 0.3 \times 10^{-6}$
CrhP	CcpA	n.d.	n.d.	$19.6 \pm 2.5 \times 10^{-6}$
HPrSerP-CcpA	*xylcre*	$2.2 \pm 0.5 \times 10^{6}$	$2.7 \pm 0.8 \times 10^{-3}$	$1.2 \pm 0.4 \times 10^{-9}$

Fluorescence and SPR measurements of the interaction of CcpA with *xylAcre* indicate only weak binding of CcpA alone to *xylAcre*, while CcpA-*xylAcre* complex formation is stimulated by HPrSerP or CrhP, but not by HPr, Crh, FBP or Glc-6-P. Titrations of *xylAcre* with HPrSerP at fixed CcpA concentrations show saturation of *cre* binding at 50 μM HPrSerP. The analogous experiment with CrhP requires more than 200 μM of CrhP for saturation of CrhP-CcpA binding to *cre*. These results demonstrate that occupation of *xylAcre* is directly linked to the extent of CcpA-HPrSerP or CcpA-CrhP complex formation.

Mixtures of FBP or Glc-6-P with HPrSerP stimulate CcpA binding to *xylAcre* at HPrSerP concentrations clearly below 50 μM indicating that FBP and Glc-6-P stimulate CcpA-DNA interaction by increasing the amount of *cre* binding competent CcpA in the HPrSerP-FBP-CcpA complex. In contrast, CrhP-CcpA binding to *xylAcre* is not affected by FBP or Glc-6-P.

The equilibrium constants for HPrSerP-CcpA binding to *xylAcre* were determined by SPR in the presence of 50 μM HPrSerP (Fig. 2*C*) and for HPrSerP-FBP-CcpA binding to *xylAcre* in the presence of 5 μM HPrSerP. The almost equal results for the kinetic and equilibrium constants of HPrSerP-CcpA and HPrSerP-FBP-CcpA complex formation with *xylAcre* (Tab. 1) suggest that FBP decreases the amount of HPrSerP necessary to accomplish full repression by CcpA (Seidel et al. 2005).

The fast kinetics of HPrSerP-CcpA interaction allows CCR to respond very quickly to changing carbon sources in the environment. The low equilibrium constant of HPrSerP-CcpA complex formation is adapted to the 1 μM CcpA and 0.1–2 mM HPrSerP levels found in Bacilli and Streptococci in the presence of glucose (Mijakovic et al. 2002, Thevenot et al. 1995, Vadeboncoeur et al. 2000). In contrast, intracellular Crh and CrhP levels are low in the presence of typical PTS sugars (Singh et al. 2008) which contributes to its small influence on CCR. It is conceivable that Crh may be more highly expressed under other conditions and/or may require other, as of yet unknown, cofactors. The rate and equilibrium constants for DNA binding of the HPrSerP-CcpA complex are similar to those of other LacI/GalR family regulators, such as PurR (Xu et al. 1998) and LacI (Bondeson et al. 1993). However, it seems that there are different types of CcpA–*cre* interactions. In the absence of cofactors, CcpA binding to the *cre* sites at the *xylA*, *xynP* (Galinier et al. 1999), *pta* (Presecan-Siedel et al. 1999), *glpFK* (Darbon et al. 2002) or *gnt* (Fujita et al. 1995) promoters is very weak, but it seems to bind strongly to the sites in *amyO* (Kim et al. 1998) and *rocG* (Belitsky et al. 2004). Moreover, CcpA also shows differential regulation of genes in log, transition and stationary phases which may result from as of yet unknown cofactors modulating CcpA activity (Lulko et al. 2007). Thus, several *cre* sequences may respond in different manners to HPrSerP, CrhP, FBP or Glc6-P mediated stimulation.

2.2 The Role of Residues His15 and Arg17 of HPr and Crh for Signaling

Important sequence differences between HPr and Crh occur at positions 15 and 17, which are the phosphorylation site (His15) in HPr and participate in recognition of EI and EII (Arg17) in the PTS (Herzberg 1992, Herzberg et al. 1992). Therefore, residue His15 in *B. megaterium* HPr was mutated to Ala to analyze the role of the imidazole side chain or to Glu to mimic the presence of phosphate at this position. Furthermore, His15 of *B. subtilis* HPr was replaced by Gln, the residue present in Crh at this position, while the Gln15 of Crh was replaced by His. The impacts of charge and length of the side chain for residue Arg17 of HPr from *B.*

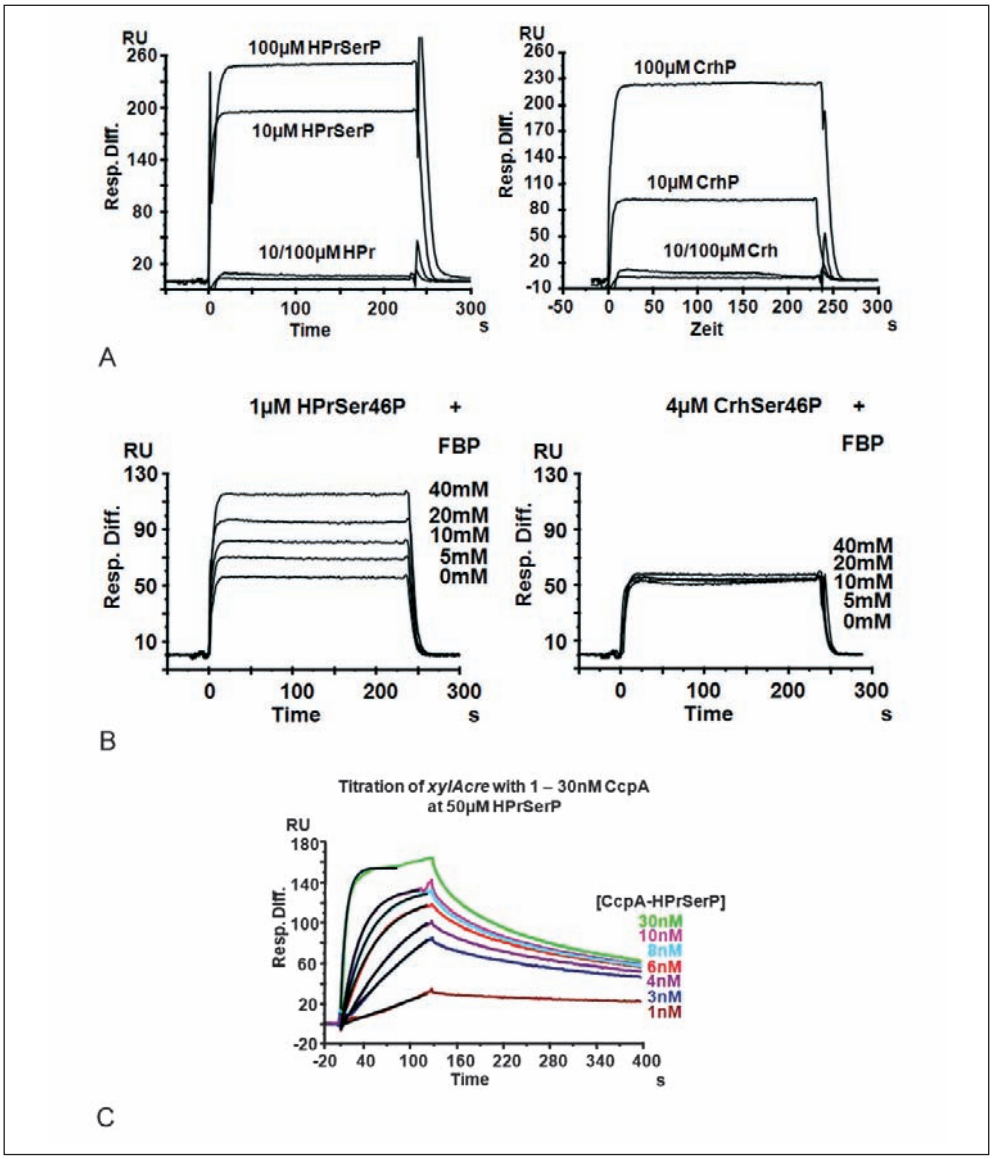

Fig. 2 Surface plasmon resonance analyses of effector binding to CcpA. (*A*) Interaction analysis of CcpA with HPr, HPrSerP, Crh and CrhP. 10 µM and 100 µM dilutions of each protein were analyzed on a chip loaded with TetR (control) in flowcell 1 and CcpA in flowcell 2. *Left diagram*: SPR analysis of HPr or HPrSerP. *Right diagram*: SPR analysis of Crh or CrhSerP. (*B*) SPR analyses of the effects of FBP on HPrSerP and CrhP binding to CcpA on a chip with TetR in flowcell 1 and CcpA in flowcell 2. *Left diagram*: titration of CcpA with mixtures of 1 µM HPrSerP and increasing concentrations (5 – 40 mM) of FBP. *Right diagram*: titration of CcpA with mixtures of 4 µM CrhP and increasing concentrations (5 – 40 mM) of FBP. (*C*) Kinetic analysis of CcpA-HPrSerP binding to *xylAcre* by SPR. The rate constants were determined from titrations of *xylAcre* with mixtures of 1 – 30 nM *B. subtilis* CcpA and 50 µM HPrSerP (running buffer with 50 µM HPrSerP). The concentrations of the CcpA-HPrSerP complex are assumed the same as those of CcpA and are depicted in the respective color. The fits of the association phases are drawn as black lines.

megaterium were investigated using HPrR17A and HPrR17K. The activities of these mutants showed that the mutations of His15 to Ala and Gln in HPrSerP, as well as the mutation of Gln15 to His in CrhP, have almost no effect on interaction with CcpA (Fig. 3). In contrast, the HPrH15ESerP interaction with CcpA was strongly impaired suggesting that histidine phosphorylation interferes with CcpA binding (Fig. 3). Another outcome of mutation of HPr at His15 to Ala or Gln is the loss of FBP/Glc-6-P stimulation of HPrSerP binding to CcpA. The respective substitution of Gln15 for His makes mutated Crh sensitive to stimulation of CrhP-CcpA binding by FBP or Glc-6P (for details see Horstmann et al. 2007). These results show that there are two modes of action of residue 15 in HPr: (*i*) The contact of His15 of HPrSerP to CcpA acts as a sensor for PTS activity via His15 phosphorylation and (*ii*) His15 is a determinant for the stimulation of HPrSerP-CcpA complex formation by FBP and Glc-6-P.

The conservative replacement of residue Arg17 by Lys in HPrR17KSerP shows only a slight influence on the interaction with CcpA while the mutant HPrR17ASerP was impaired in complex formation (Fig. 3). HPrH15ASerP, HPrH15QSerP, and CrhQ15HSerP stimulated *cre* binding of CcpA as efficiently as the respective wild types while the HPrH15ESerP and HPrR17ASerP mutants did not (Fig. 3). The HPrR17KSerP stimulated *cre* binding only poorly, although it bound CcpA very well (Fig. 3). This demonstrates that residue R17 of HPr is also essential for triggering the structural change of CcpA into its *cre* binding form. Hence, there are two distinct roles of Arg17: (*i*) binding to CcpA as demonstrated by the reduced affinity of HPrR17ASerP for CcpA accompanied by the lack of *cre* binding and (*ii*) promoting the structural change of CcpA into the *cre*-binding conformation as indicated by the nearly wild type affinity of HPrR17KSerP for CcpA and the lack of *cre* binding of the formed complex. The latter result can be explained on the basis of the x-ray structures of the apo and DNA bound forms of CcpA. The N-terminal core subdomains of the opposing monomers rotate toward each other to form the DNA-binding conformation. In the HPrSerP-CcpA-*cre* complexes HPrSerP bridges the N-terminal subdomains in the CcpA dimer by binding three Asp residues, one of CcpA1 and two of CcpA2 (Horstmann et al. 2007, Schumacher et al. 2004). In the R17K mutant Lys17 can only contact the Asp residue of monomer CcpA1, not those of CcpA2, and hence cannot support the DNA-binding structure. Our results clearly demonstrate that this bridging of the CcpA dimers by Arg17 of bound HPrSerP is necessary for binding of the complex to *cre*.

3. Conclusion

In conclusion, CcpA is a rather untypical member of the LacI/GalR family of bacterial regulators. Effectors and regulatory determinants are multifaceted and are summarized together with the respective K_D values in a scheme of the CcpA regulatory circuit in Figure 4.

While CcpA undergoes similar conformational changes between the DNA-binding and non-binding forms just like other members of the LacI/GalR family, it makes use of different effectors binding to different sites of the protein to accomplish this task. Binding of a typical LacI/GalR-like effector (FBP or Glc-6-P) into the effector binding cleft of CcpA leads to an increase of affinity for HPrSerP, which in turn triggers the allosterical conformational change necessary for *cre* binding. Moreover, besides HPrSerP, CrhP, FBP and Glc-6P there are more interaction partners expected, e.g. for activation of transcription by CcpA. During the last decade many groups showed that AT-rich Gram-positive bacteria use these complex interde-

pendencies of regulation by CcpA to adapt the expression of genes with distinct functionalities involved in carbon catabolism, amino acid anabolism, overflow metabolism, competence, sporulation and biofilm formation. This versatility in effector and *cre* recognition seems to

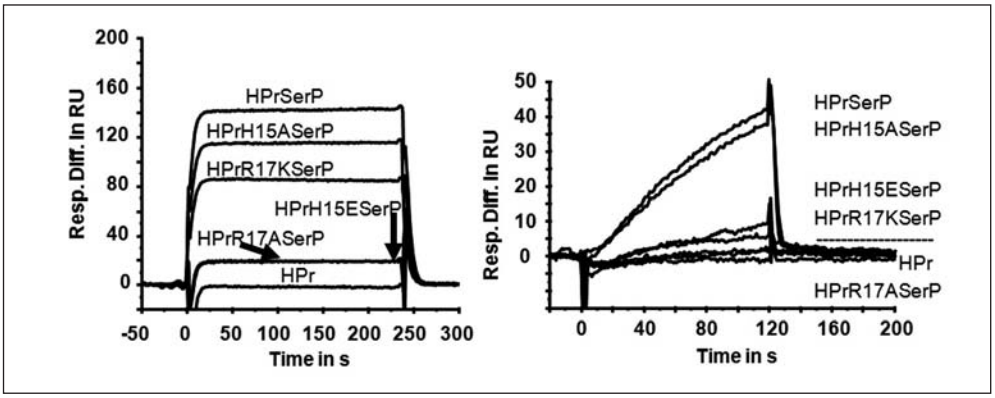

Fig. 3 Qualitative SPR analyses of HPr, HPrSerP and HPrSerP(mutant) interactions their regulatory effects. Left diagram: HPr, HPrSerP and HPrSerP(mutant) binding to CcpA. 100 µM HPr and 15 µM each from wtHPrSerP and HPrSerP mutant proteins from *B. megaterium* were analyzed on a chip with TetR immobilized in flowcell 1 and CcpA(His_6) from *B. megaterium* in flowcell 2. HPr species are depicted above each sensorgram. Right diagram: SPR analysis of HPrSerP(mutant)-CcpA complexes binding to cre. Sensorgrams were obtained from the interaction analysis of *cre* with complexes formed from CcpA(His_6) (10 nM) in the presence of 15 µM of HPr, HPr-Ser46-P or different HPr mutants from *B. megaterium* as depicted next to each sensorgram.

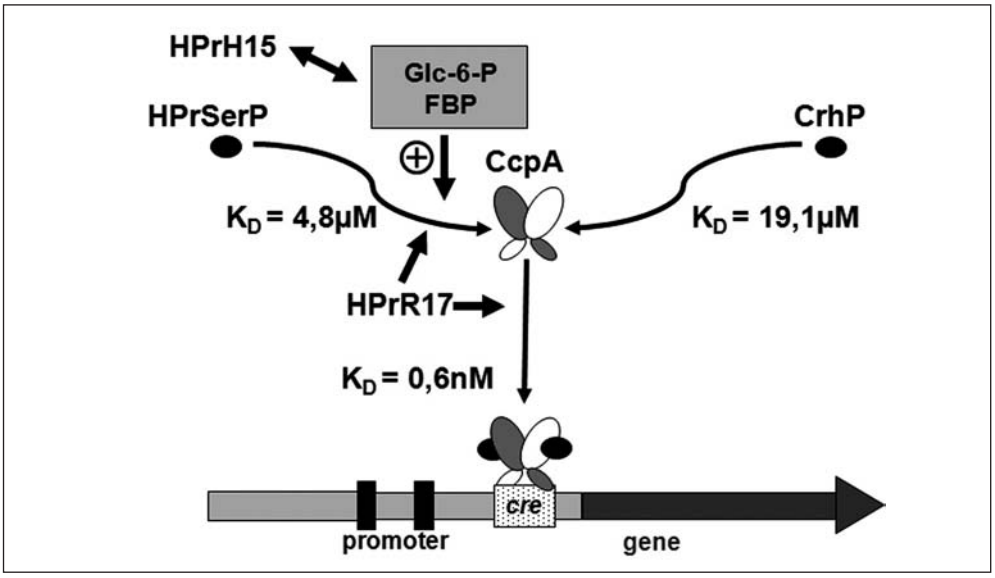

Fig. 4 Schematic summary of the regulatory circuit of CcpA. Interactions are depicted as thin arrows and bold arrows point out regulatory influence of FBP and Glc-6-P or of residues H15 or R17 of HPr on the respective interactions. Equilibrium constants near thin arrows illustrate quantitative interdependencies.

be the prerequisite of CcpA for differential regulation of genes dependent on growth conditions and growth phases, making CcpA a regulator responsive to different environmental and physiologic conditions of the cell. Among pathogens Streptococci, *Staphylococcus aureus* and *Clostridium perfringens* use this capability of CcpA for direct virulence gene regulation (Abranches et al. 2008, Hondorp and McIver 2007, Kinkel and McIver 2008, Seidl et al. 2008).

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Continents under Climate Change

Konferenz aus Anlass des 200. Gründungsjubiläums der Humboldt-Universität zu Berlin

in Zusammenarbeit mit dem Potsdam-Institut für Klimafolgenforschung (PIK) und der Deutschen Akademie der Naturforscher Leopoldina

Unter der Schirmherrschaft des Auswärtigen Amtes der Bundesrepublik Deutschland

vom 21. bis 23. April 2010 in Berlin

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Der Klimawandel gehört zu den drängendsten globalen Problemen unserer Zeit. Die Menschheit steht vor besonderen Herausforderungen, um insbesondere den CO_2-Ausstoß zu senken. Führende Wissenschaftler aus der Klimaforschung betrachten die Auswirkungen des Klimawandels auf die Kontinente Europa, Asien, Afrika, Amerika und Australien sowie die Polarregionen. Dabei werden neueste Klimadaten unter globalen und regionalen Gesichtspunkten ausgewertet und Simulationsmodelle für zukünftige Entwicklungen diskutiert. Die Ausführungen bieten ein gut fundiertes Bild der Klimaänderungen, die sich weltweit bereits vollziehen bzw. in Zukunft ereignen werden, und untersuchen kritisch die Folgen für Natur, Gesellschaft und Wirtschaft. Der Kongress „Continents under Climate Change“ wurde im Rahmen der 200. Jahrfeier der Humboldt-Universität zu Berlin vom Potsdam-Institut für Klimaforschung und der Deutschen Akademie der Naturforscher Leopoldina – Nationale Akademie der Wissenschaften veranstaltet. Alle Beiträge sind in englischer Sprache verfasst.

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Temperature-dependent Proteolysis as a Control Element in Bacterial Metabolism

Aviram Rasouly, Eyal Gur, Chen Katz, Yotam Shenhar, Dvora Biran, and Eliora Z. Ron (Tel Aviv, Israel)

Abstract

An increase in temperature results in major physiological changes, as enzymes work faster but proteins tend to unfold. Consequently, upon a shift-up in temperature several regulatory response mechanisms are induced to maintain balanced growth. One important mechanism involves temperature-dependent proteolysis. Here we discuss the effect of proteolysis on three essential pathways – protein synthesis, synthesis of lipopolysaccharides (LPS) and the heat shock response.

Zusammenfassung

Ein Anstieg der Wachstumstemperatur führt bei *Escherichia coli* zu wichtigen physiologischen Änderungen, da einerseits die Aktivität der Enzyme steigt und andererseits Proteine durch Denaturierung ihre Funktion verlieren. Folglich müssen bei Temperaturänderungen Regelmechanismen für ein ausbalanciertes mikrobielles Wachstum sorgen. Ein wichtiger Regelmechanismus ist die temperaturabhängige Proteolyse. In diesem Beitrag wird der Effekt der Proteolyse an drei wichtigen Schnittstellen des Metabolismus aufgezeigt: Proteinsynthese, Lipopolysaccharidsynthese und Hitzeschockantwort.

1. Introduction

Bacteria grow in a wide range of temperatures. Mesophilic bacteria, such as *Escherichia coli*, can grow at temperatures ranging from less than 20 °C to as high as 45 °C. A shift-up in temperature brings about a major physiological change due to the fact that enzymes work faster, but also tend to unfold. The activity of the proteins is maintained by the protein quality control system. This system includes chaperones that refold unfolded proteins and proteases, which degrade proteins that failed to resume their native form. Here we discuss three proteolytic systems that are highly significant for balanced growth at elevated temperatures.

2. Control of Protein Biosynthesis

Upon a shift to a temperature higher than 30 °C, *E. coli* culture growing in minimal media immediately assumes a new growth rate typical of the new temperature (Ron and Davis 1975). This change in growth rate as a function of temperature is mediated by the first enzyme in methionine biosynthesis – homoserine trans-succinylase (HTS) (Ron and Shani

1971, Ron et al. 1990). This enzyme is extremely thermolabile and undergoes unfolding at temperatures above 25 °C. The activity of HTS decreases, and at 46 °C the enzyme is inactive and aggregated, resulting in an absolute requirement for methionine (Gur et al. 2002). An additional factor in the temperature-sensitive regulation of methionine biosynthesis is the fact that HTS is an unstable enzyme which undergoes proteolysis at a rate that increases with temperature (Biran et al. 2000). The degradation of HTS is carried out by ATP-dependent proteolysis (Biran et al. 2000) and provides an additional control level for the availability of methionine and the rate of growth at elevated temperatures.

The limitation of the availability of methionine has several consequences – protein synthesis is blocked at initiation, RNA synthesis is inhibited by the stringent response, and DNA synthesis is inhibited at initiation leading to inhibition of cell division. There is also an inhibition of the transfer of C1-fragments (important in a large number of pathways, including the biosynthesis of purines and pyrimidines) and inhibition of the synthesis of polyamines (spermidine and spermine). Thus, it is clear that regulating growth rate by proteolysis and inactivation of the first enzyme in methionine biosynthesis will have an immediate effect on a large number of metablic pathways including the synthesis of all the macromolecules.

3. Control of LPS Synthesis

Lipopolysaccharides (LPS) are a major cellular component of Gram-negative bacteria and have several important physiological roles. The minimal essential unit of LPS is anchored to the membrane and is composed of lipid-A bound to two units of keto-deoxy-octonate (KDO). This unit is essential, and mutants in its synthesis are lethal. Long polysaccharide units (the O-antigen) are attached to the lipidA-KDO complex forming the complete LPS. Mutants defective in the synthesis of the O-antigen ("rough mutants") are viable, but are altered in their permeability, especially for hydrophobic compounds, and are therefore very sensitive to hydrophobic antibacterial drugs, such as polyketides. In addition, rough mutants are extremely sensitive to phagocytosis and to the complement system and are therefore avirulent.

An essential protein that catalyzes the second reaction and the first committed step in the biosynthesis of lipid A is UDP-3-O-acyl-N-acetylglucosamine deacetylase (LpxC). Previous studies indicated that LpxC is an unstable protein that is degraded by FtsH, the membrane bound, essential, ATP-dependent protease (Herman et al. 1995, Tatsuta et al. 1998, Tomoyasu et al. 1995). The accumulation of LpxC results in an unbalanced ratio of lipid A (in the biosynthesis of lipopolysaccharides [LPS]) to phospholipids, as both pathways use the same precursor (-3-hydroxymyristoyl-acyl carrier protein), which is present in limited amounts (Ogura et al. 1999).

In *ftsH* mutant LpxC accumulates, leading to increased synthesis of lipidA, a decreased synthesis of phospholipids and eventual cell death. Thus, the FtsH protease controls the balance between phospholipids and the lipid A moiety of lipopolysaccharides (Ogura et al. 1999). As FtsH is a heat shock protein, whose cellular levels increase significantly upon temperature shift-ups, this regulation of LPS synthesis is affected by temperature.

Recent results demonstrated an additional role for FtsH in regulating LPS biosynthesis. Thus, FtsH degrades 3-deoxy-D-manno-octulosonate (KDO) transferase (KdtA) which carries out the attachment of two KDO residues to the lipid A precursor (lipid IVA) to form the minimal essential structure of the lipopolysaccharide (LPS) (KDO2-lipid A). Thus, FtsH

regulates the concentration of the lipid moiety of LPS (lipid A) as well as the sugar moiety (KDO-based core oligosaccharides), ensuring a balanced synthesis of LPS (Katz and Ron 2008).

4. Proteolysis as a Control Element in the Heat Shock Response

In *E. coli*, the heat shock genes are transcribed from specific promoters by the heat shock transcriptional activator σ32, encoded by the *rpoH* gene. At low temperatures, σ32 levels are kept low by the proteolytic activity of FtsH. Thus, proteolysis constitutes a major factor in the regulation of the heat shock response (Arsene et al. 2000, Herman et al. 1995, Kanemori et al. 1999, Tatsuta et al. 1998, 2000, Tomoyasu et al. 1995).

Proteolysis has an additional function in regulating the heat shock response, as it also plays an important role in the shutoff of this response. One unique feature of the heat shock response is the kinetics of induction of the heat shock genes. The expression of the heat shock genes increases rapidly but also declines rapidly, although the stress factor still exists. It was shown that the decrease in expression of heat shock genes correlates with a considerable reduction in the half life of the transcripts of these genes. It was demonstrated that transcripts of many σ32-dependent genes are stabilized by the RNA chaperone CspC (Cold shock protein C). Upon shifts to high temperatures the levels of CspC are reduced, due to proteolysis and aggregation, leading to a decrease in the stability of mRNAs of heat shock genes, which now have a shorter half life. Overexpression of CspC eliminates the rapid shut-off of expression of the heat shock genes and abolishes its transient nature (Shenhar et al. 2009).

Most *E. coli* proteins are stable and the ones with a half life shorter than the generation time are usually regulatory proteins present in small concentrations (i.e., SulA, the division inhibitor or σ32, the transcriptional activator of the heat shock response). The group of proteins presented here have physiological functions under all growth conditions and are present at high levels under conditions of balanced growth. Yet, upon temperature increases these proteins are proteolysis substrates and their reduced level has major effects on the expression of central physiological systems.

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RNA Biology in Bacterial Metabolism and Virulence

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A Small RNA Cascade Regulates Aminosugar Synthesis

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With 3 Figures

Abstract

Small noncoding RNAs (sRNAs) and the RNA chaperone, Hfq, have increasingly been implicated as post-transcriptional regulators of bacterial sugar pathways. Recent investigations of the Hfq-dependent GlmY and GlmZ sRNAs in *Escherichia coli* have discovered a novel mechanism to differentially activate *glmS* mRNA coding for an essential enzyme in amino sugar metabolism. The two sRNAs, although highly similar in sequence and structure, act in a hierarchical manner and form a complex regulatory cascade with multiple input functions. The GlmYZ-mediated discoordinated expression of the *glmUS* operon constitutes the first case of post-transcriptional control by sRNAs that leads to activation of a downstream cistron. The data also suggests that Gram-negative bacteria evolved a mechanism of *glmS* riboregulation that is distinct from the *glmS* ribozyme mechanism of Gram-positive bacteria.

Zusammenfassung

Kleine nichkodierende RNAs (sRNAs) und das RNA-Chaperon Hfq spielen zunehmend eine Rolle für unser Verständnis der post-trankriptionellen Genregulation im Zuckerstoffwechsel von Bakterien. Wir haben kürzlich bei der Untersuchung der weit verbreiteten Hfq-abhängigen GlmY und GlmZ sRNAs in *Escherichia coli* ein neues Prinzip der Genregulation auf der RNA-Ebene entdeckt. GlmY und GlmZ fungieren beide als Aktivatoren der Expression der *glmS* mRNA, die ein essentielles Enzym im Aminozucker-Stoffwechsel kodiert. Obgleich die beiden sRNAs in ihrer Sequenz und Struktur sich sehr ähnlich sind, ist ihre Funktion nicht redundant; im Gegenteil, ähnlich Transkriptionsfaktoren agieren GlmY und GlmZ in hierarchischer Art und bilden das Herz einer komplexen regulatorischen Regulationskaskade mit vielfältigen Eingangssignalen. Unsere Untersuchung von GlmYZ hat zudem zum ersten Mal gezeigt, dass durch sRNAs sehr selektiv ein individuelles Cistron eines Operons post-transkriptionell aktiviert werden kann. Insgesamt unterscheidet sich das GlmYZ-System zur Kontrolle der Synthese von GlmS fundamental von der zuvor beschriebenen Regulation durch ein Ribozym in Gram-positiven Bakterien.

1. Introduction

Because all organisms depend on carbohydrates which provide cells with energy and the building blocks for biosynthesis of all macromolecules, it is not surprising that the uptake and metabolism of carbohydrates is extensively regulated at all levels. The genes required for the utilization of a particular sugar substrate are typically expressed only if it is available in the environment, as best demonstrated for regulation of the *Escherichia coli* lactose operon by the Lac repressor (MÜLLER-HILL 1996). Nonetheless, substrate-dependent regulation of gene expression also occurs beyond the level of transcriptional initiation by a variety of mechanisms, including transcriptional attenuation, translational control, and modulation of mRNA decay.

Historically, the study of bacterial gene regulation has been focused on regulatory proteins. However, the recent discoveries of unexpected numbers of small noncoding RNAs (sRNAs) and novel *cis*-encoded RNA control elements have challenged the perception that proteins are the only relevant players in the control of gene expression. Bacterial sRNAs are typically 50–250 nucleotides in length, generally untranslated, and encoded in the "empty" intergenic regions of bacterial chromosomes (Vogel and Sharma 2005). Most sRNAs are specifically regulated by a cognate transcription factor under certain growth or stress conditions, and function as regulators by base-pairing with *trans*-encoded mRNAs, thereby either repressing or activating target genes at the post-transcriptional level. Some sRNAs specifically modulate protein functions (Majdalani et al. 2005, Waters and Storz 2009).

Noncoding RNA also regulates gene expression in *cis*. This type of regulation includes the well-known class of antisense RNAs encoded opposite to mRNA genes (Wagner et al. 2002), and the classes of riboswitches and RNA thermometers. Riboswitches, which are highly structured RNA elements found in 5' untranslated regions (5' UTRs) of metabolic genes, typically sense the metabolite that is synthesized or taken up by the downstream encoded protein(s). Binding of the metabolite entails a change in RNA structure, which then facilitates feedback regulation at the transcriptional or translational level (Winkler and Breaker 2005). RNA thermometers are RNA structures whose formation is highly responsive to environmental temperature. These elements sequester the ribosome-binding site (RBS) of their host mRNA, and their temperature-dependent melting permits control of mRNA translation (usually activation) (Narberhaus et al. 2006).

To date, sRNAs have been most intensely studied in the enterobacterial model organisms, *E. coli* and *Salmonella*, in which a variety of approaches (Altuvia 2007, Vogel and Sharma 2005, Vogel 2009) have identified more than a hundred chromosomally encoded sRNAs. In contrast, *Bacillus subtilis* has been the organism of choice for the study of riboswitches; approximately 5 % of all genes of this soil bacterium might be controlled by RNA-based metabolite sensing (Mandal et al. 2003). In other words, riboswitches are abundant in low G+C Gram-positive bacteria such as *B. subtilis*, but rare in *E. coli* (Barrick and Breaker 2007). Reciprocally, *trans*-encoded sRNAs are abundant in enteric bacteria, and it is therefore tempting to speculate that sRNAs generally compensate for the paucity of riboswitch-mediated control in these species.

The recent discoveries of fundamental differences in *glmS* regulation in *B. subtilis* (ribozyme) and *E. coli* (sRNAs) support the above speculation. The Breaker laboratory discovered a novel riboswitch mechanism that controls the *Bacillus subtilis glmS* mRNA. Its 5' UTR contains a ribozyme that undergoes self-cleavage in the presence of GlcN-6-P (glucosamine-6-phosphate), thereby destabilizing the *glmS* mRNA (Winkler et al. 2004). This *cis*-encoded mechanism of riboregulation seems highly conserved among Gram-positive bacteria (Barrick et al. 2004), yet no amino sugar-induced cleavage was observed with the *E. coli glmUS* IGR (E. Lee and R. R. Breaker, personal communication).

In *E. coli*, the *glmUS* operon encodes two enzymes of the biosynthetic amino sugar pathway for GlcN (glucosamine) and its derivative GlcNAc (*N*-acetylglucosamine) (Fig. 1). Both amino sugars are required as precursors for the synthesis of peptidoglycan and lipopolysaccharide, essential components of the cell wall of Gram-negative bacteria. GlmS is a GlcN synthase that converts fructose-6-P to GlcN-6-P using glutamine as an amino donor; GlmU is a bifunctional enzyme that sequentially converts GlcN-1-P to UDP-GlcNAc. Whereas GlmU is constantly required because its substrate derives from both external and internal

sources, GlmS is only essential when insufficient external aminosugar supply requires intracellular synthesis of GlcN-6-P from fructose and glutamine. The two promoters that control the *glmUS* operon are both located upstream of *glmU*, implying strict co-transcription of the *glmU* and *glmS* genes (Plumbridge 1995). Therefore, the relative synthesis of GlmS in response to substrate availability can only be adjusted at the post-transcriptional level. We will describe below how this is achieved by two sRNAs, GlmY and GlmZ, in *E. coli* (Fig. 2*A*).

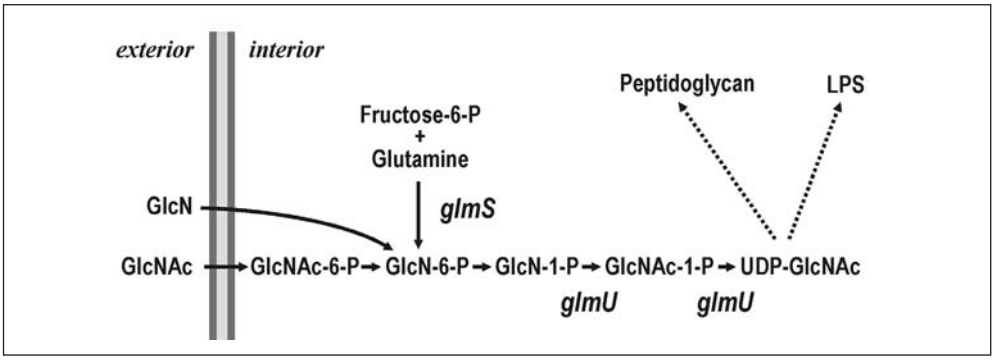

Fig. 1 Enzymatic steps in amino sugar synthesis that are catalyzed by the *glmU* and *glmS* gene products in *E. coli* (adapted from Plumbridge 1995).

2. The GlmYZ sRNAs Activate the Downstream Cistron in the *glmUS* mRNA

Following transcription of the dicistronic *glmUS* operon, RNase E-dependent processing generates a monocistronic *glmS* (Joanny et al. 2007, Kalamorz et al. 2007). The cleavage itself does not appear to be metabolically regulated; instead, sub-operonic stimulation of GlmS synthesis is mediated by GlmY and GlmZ (Kalamorz et al. 2007, Reichenbach et al. 2008, Urban et al. 2007, Urban and Vogel 2008).

The *E. coli* GlmY and GlmZ sRNAs (a.k.a. SroF/tke1 and RyiA/SraJ, respectively) were originally discovered in several of the genome-wide screens that led to a full appreciation of the abundance of sRNA genes in bacteria (Argaman et al. 2001, Rivas et al. 2001, Vogel et al. 2003, Wassarman et al. 2001). Their cellular role, as well as their now obvious functional and structural relationship, was not realized before either of them was found to activate GlmS synthesis. Overexpression of GlmY was observed to cause GlmS accumulation to an extent that this protein was readily visible on standard SDS gels. In contrast, the level of the upstream encoded GlmU protein was not increased (Urban et al. 2007). Independent work from the Görke laboratory identified the *glmZ* gene as the cause of chronic GlmS overproduction in a certain *E. coli* mutant (*yhbJ*; see below); again, only GlmS and not GlmU synthesis was altered (Kalamorz et al. 2007).

Together, this was the first case in which sRNAs had mediated post-transcriptional activation of protein synthesis within a polycistronic messenger. Prior to that, the *galETKM* mRNA had been reported to be subject to post-transcriptional "discoordinate regulation" by the *trans*-encoded Spot42 sRNA. However, Spot42 represses the synthesis of the downstream encoded GalK (by masking the *galK* RBS) whilst the upstream encoded GalE and GalT proteins are not affected (Møller et al. 2002).

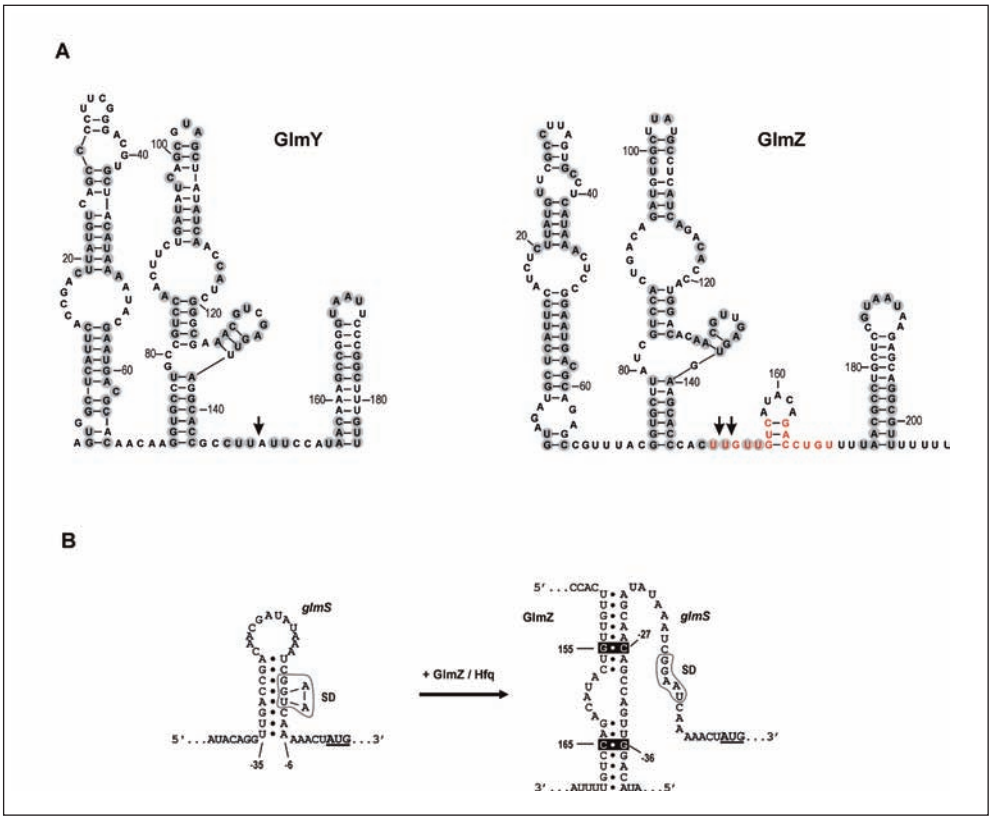

Fig. 2 The GlmY and GlmZ sRNAs, and the mechanism of direct *glmS* activation. (*A*) Consensus structures of the *E. coli* GlmY (184 nt) and GlmZ (207 nt) RNAs based on sequence alignments (published in URBAN et al. 2007, URBAN and VOGEL 2008). Vertical arrows indicate previously mapped 3' processing sites (ARGAMAN et al. 2001, VOGEL et al. 2003). Grey circles denote nucleotides conserved between the *E. coli* GlmY and GlmZ RNAs. The GlmZ residues involved in *glmS* mRNA binding (see Fig. 2*B*) are shown in red. (*B*) Predicted anti-antisense mechanism by which GlmZ activates the *E. coli glmS* mRNA. The left panel shows the 5' UTR of the *glmS* mRNA in an unbound, "inactive" state, in which an intrinsic hairpin sequesters the *glmS* SD (boxed) and thus inhibits translation. Residues −42 to +3 relative to AUG start codon, which is underlined, are shown. The right panel shows *glmS* mRNA in its "activated" form, i.e. upon base-pairing of residues 150–157 and 163–169 of GlmZ RNA with the *glmS* 5' UTR, which disrupts the inhibitory hairpin and liberates the *glmS* SD. The positions of point mutations introduced in *glmS* (*glmS** allele) and GlmZ (GlmZ* allele), which maintain base-pairing in the *glmS**/GlmZ* duplex, are indicated by inverse print.

3. Translational Activation of *glmS* mRNA by GlmZ

GlmY and GlmZ are highly similar in length, sequence and structure (Fig. 2*A*). However, these seemingly homologous sRNAs activate GlmS synthesis by entirely different mechanisms and act hierarchically in noncoding RNA cascade in which only GlmZ directly interacts with the target mRNA. By default, translation of *glmS* is weak owing to an internal hairpin structure that sequesters the Shine-Dalgarno (SD) sequence (Fig. 2*B*) and limits ribosome access to this messenger. This configuration is reminiscent of the *rpoS* mRNA encoding the general stress sigma factor, σS, of *Salmonella* and *E. coli*. The Gottesman and Belfort laboratories showed

that *rpoS* translation is activated in *E. coli* by two *trans*-encoded sRNAs, DsrA and RprA. Pairing of either sRNA to the 5' flank of the inhibitory hairpin induces a structural rearrangement of *rpoS* mRNA, thereby rendering the RBS accessible and promoting *rpoS* translation (Lease et al. 1998, Majdalani et al. 1998, 2002).

RNA structure probing experiments (Urban and Vogel 2008) and *in silico* analysis (Kalamorz et al. 2007) hinted at the same mechanism for *glmS*: Approximately fifteen residues of a single-stranded region of GlmZ RNA (between stem-loops 2 and 3) target the 5' flank of the *glmS* RBS hairpin (Fig. 2*B*) to liberate the SD. This model has been experimentally validated by the successful introduction of compensatory basepair changes in the interacting GlmZ and *glmS* RNAs. Specifically, point mutations in the upper and lower helices of the GlmZ-*glmS* interaction abrogate *glmS* induction, but when combined in a compensatory fashion, activation by GlmZ is restored (Urban and Vogel 2008). Moreover, experiments using purified 70 S ribosomes recapitulated the *glmS* mRNA activation by GlmZ *in vitro* (Urban and Vogel 2008), demonstrating that this "anti-antisense mechanism" requires only the translational machinery and an activator sRNA. Because of additional examples from *E. coli*, *Vibrio* and *Staphylococcus* species (Hammer and Bassler 2007, Morfeldt et al. 1995, Prevost et al. 2007), translational activation by anti-antisense pairing of sRNAs is now regarded as one of the principle mechanisms of bacterial riboregulation.

The GlmZ-mediated activation of *glmS* translation also stabilizes this mRNA (Kalamorz et al. 2007, Urban and Vogel 2008), likely because of the well-established synteny of transcription and translation that generally protects bacterial messengers from degradation (Deana and Belasco 2005, Dreyfus 2009). In addition, we note that, *in vitro*, the addition of purified Hfq protein increases the effect of GlmZ on translation almost tenfold, whereas the protein by itself acts as a translational repressor (Urban and Vogel 2008). Hfq is also required for *glmS* activation *in vivo* (Kalamorz et al. 2007, Urban and Vogel 2008), and associates with both the GlmY or GlmZ sRNAs (Sittka et al. 2008, Zhang et al. 2003) and with the *glmUS* intergenic region (Sittka et al. 2008). Thus, Hfq is a major player in this regulation.

4. A Regulatory RNA Cascade – GlmY Acts upon GlmZ to Activate GlmS Synthesis

As previously mentioned, the GlmY sRNA is almost identical in appearance to GlmZ. However, it lacks the residues by which GlmZ targets the *glmS* hairpin (Fig. 2*A*). Moreover, GlmY failed to promote GlmS synthesis *in vitro* (Urban and Vogel 2008), which suggests that it might not be a direct regulator. If this is the case, how does GlmY activate GlmS synthesis *in vivo*?

The full-length (207 nt) GlmZ sRNA is subject to 3' RNA processing that involves the – direct or indirect – action of YhbJ, a conserved protein of as yet unknown function (Kalamorz et al. 2007). Processing generates a shorter (153 nt) GlmZ RNA (Argaman et al. 2001) which loses the *glmS* targeting region and therefore fails to promote GlmS synthesis *in vitro* (Urban and Vogel 2008). Presumably by RNA mimicry, GlmY can antagonize the GlmZ processing and thus increase the levels of the direct *glmS* activator (Reichenbach et al. 2008, Urban and Vogel 2008). Importantly, families of homologous sRNAs are known to exist in diverse bacteria (Axmann et al. 2005, Lapouge et al. 2008, Lenz et al. 2004, Wilderman et al. 2004). Where such families had been functionally characterized, the sRNAs were always

found to act in a redundant and/or additive manner. In stark contrast, the GlmYZ sRNAs act hierarchically, such that GlmY requires GlmZ for *glmS* activation (Reichenbach et al. 2008, Urban and Vogel 2008).

5. The Status of GlmY RNA Polyadenylation Controls Activation of GlmS Synthesis

In addition to Hfq and YhbJ, which directly engage in the GlmYZ cascade by controlling GlmZ activity at the RNA level (Kalamorz et al. 2007, Urban and Vogel 2008), the main enzyme responsible for 3' end RNA polyadenylation, PAP I (poly(A) polymerase I encoded by *pcnB*, Cao and Sarkar 1992), plays a role in the circuit. PAP I also acts at the post-transcriptional level, by reducing the stability and level of GlmY sRNA. In fact, PAP I was the first factor implicated in post-transcriptional *glmS* regulation, and was identified in an investigation of the global effects of an *E. coli pcnB* mutant. Specifically, the Hajnsdorff laboratory demonstrated that loss of PAP I function causes chronic overproduction of both *glmS* mRNA and GlmS protein (Joanny et al. 2007). Poly(A) tails at the 3' end typically destabilize bacterial transcripts, rendering RNA a better target for the activities of 3'->5' exonucleases (Dreyfus and Regnier 2002, Kushner 2004). Consequently, it was speculated that the loss of polyadenylation directly impeded the decay of *glmS* mRNA, thereby increasing GlmS synthesis (Joanny et al. 2007).

Several observations, however, prompted the re-evaluation of this model. First, *glmS* reporter fusions with 3' ends different from native *glmS* mRNA, as well as the GlmYZ sRNAs were also upregulated in the PAP I-deficient strain (*pcnB*Δ1) (Kalamorz et al. 2007, Reichenbach et al. 2008, Urban and Vogel 2008). Second, rifampicin treatment experiments and 3' RACE-mediated assessment of the poly(A) status of the GlmY, GlmZ or *glmS* RNAs demonstrated that GlmY (and not *glmS* or GlmZ) exhibited differential stability and expression in *pcnB*Δ1 cells that correlated with the presence or absence of 3' end poly(A) tails. These findings have led to a revised model such that if GlmY is no longer polyadenylated (normally > 50 % of all GlmY molecules), this sRNA is markedly stabilized. Its accumulation then stabilizes GlmZ (by antagonism of GlmZ processing) and indirectly increases *glmS* translation (Reichenbach et al. 2008, Urban and Vogel 2008).

Interestingly, polyadenylation-dependent RNA stability had long been known to play a role in plasmid copy number control by *cis*-encoded antisense RNAs (Dam Mikkelsen and Gerdes 1997, Söderbom et al. 1997, Xu et al. 1993). In contrast, GlmY was the first chromosomal sRNA whose polyadenylation had impacted the synthesis of a cellular protein with a key function in bacterial physiology.

6. A Regulatory sRNA Cascade with Multiple Entry Points?

Although it was well-established that GlmS activity was feedback-inhibited at the enzyme level in eukaryotic systems (Milewski 2002) and *cis*-controlled in Gram-positive bacteria (Winkler et al. 2004), it had remained unknown if and how this important protein was autoregulated in Gram-negative bacteria. Using various inhibitors of amino sugar and cell wall synthesis pathways, the Görke laboratory demonstrated that the GlmYZ RNAs are essential factors in the feedback control of *E. coli* GlmS synthesis by its very product, GlcN6P.

If GlcN6P is depleted, GlmY accumulates, which in turn reinforces GlmS synthesis by stabilizing the GlmZ sRNA (KALAMORZ et al. 2007, REICHENBACH et al. 2008). However, we currently know little as to how the GlmYZ RNA cascade (Fig. 3) senses the environmental signals that determine *glmS* epistasis. For example, it will be important to understand precisely how YhbJ affects GlmZ RNA processing, whether any proteins other than RNase E, Hfq and PAP I target the involved RNAs, and perhaps whether any of the observed RNA-processing events are directly controlled by metabolites.

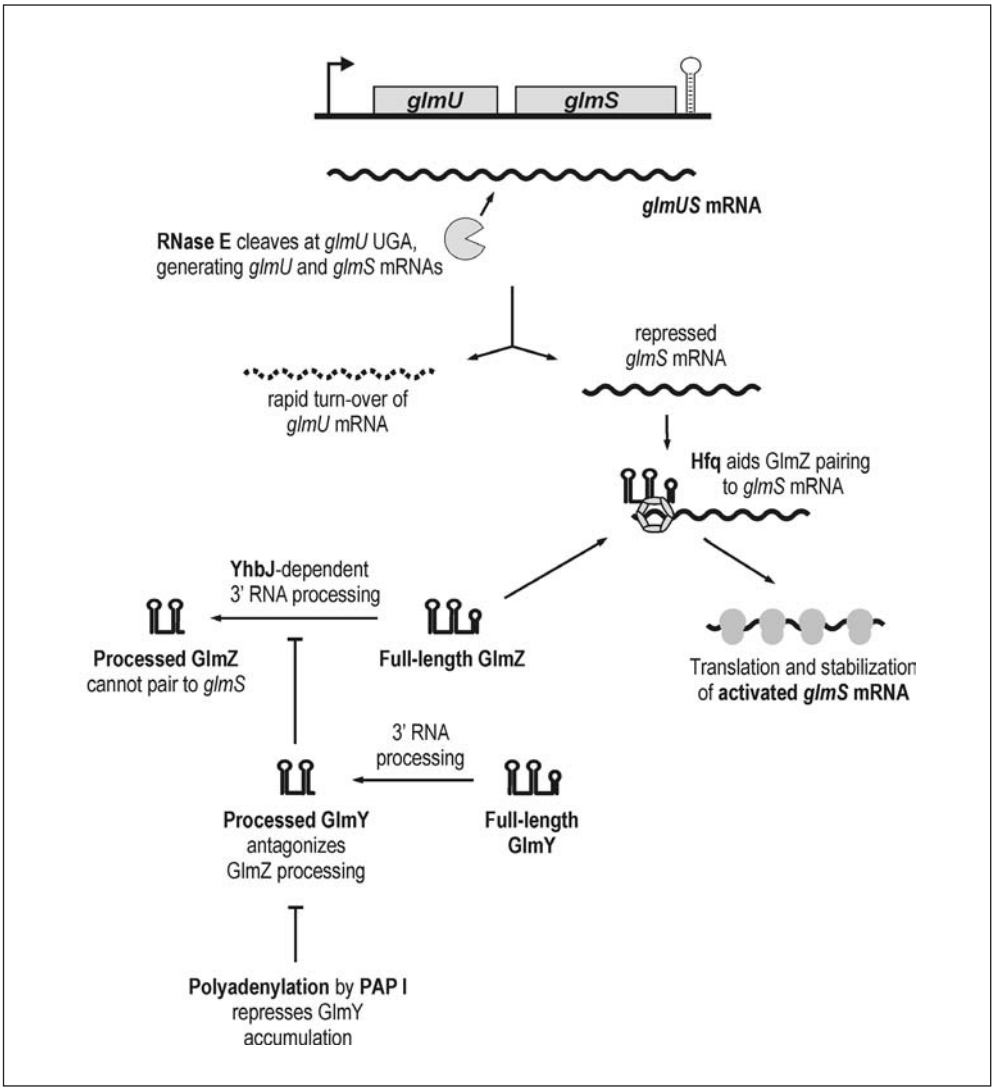

Fig. 3 Pathways of *glmS* activation by GlmY and GlmZ RNAs in *E. coli*. Model summarizing the findings by JOANNY et al. 2007, KALAMORZ et al. 2007, REICHENBACH et al. 2008, URBAN et al. 2007, URBAN and VOGEL 2008). See text for details.

7. Conclusion and Outlook

In addition to the control of virulence and transcription factors (Romby et al. 2006, Wassarman 2007) and outer membrane protein biogenesis (Guillier et al. 2006, Vogel and Papenfort 2006), the regulation of metabolic sugar pathways has emerged as a major domain of bacterial sRNA action. In addition to GlmYZ and Spot42, *E. coli* SgrS sRNA was identified as a sugar regulator; it represses the *ptsG* and other mRNAs of the PTS sugar transporters specific for glucose, fructose and mannose (Vanderpool 2007). Our analysis of SgrS functions in *Salmonella* has discovered that this sRNA also operates beyond sugar metabolism, and that it acts to repress the mRNA of horizontally acquired virulence factor under host invasion conditions (K. Papenfort and J. Vogel, in preparation). This indicates that Hfq-dependent sRNAs might cross-connect carbohydrate utilization and virulence in pathogenic bacteria.

It is worthwhile emphasizing that global transcriptome data of *hfq* mutants (Ding et al. 2004, Guisbert et al. 2007, Sittka et al. 2008, Sonnleitner et al. 2006) predict an extensive role of Hfq in the regulation of sugar metabolism. Likewise, microarray- and deep sequencing-based profiling of Hfq-associated RNA (Sittka et al. 2008, Zhang et al. 2003) showed that Hfq targets mRNAs of many sugar transporters, metabolic enzymes as well as those of transcription factors that coordinate the uptake and utilization of carbohydrates. Given the primary function of Hfq as a matchmaker of sRNA-mRNA interactions, these Hfq-associated mRNAs might as well be direct targets of regulatory sRNAs. Therefore, we might have seen just the tip of the iceberg of the manifold and widespread role for sRNAs in the control of sugar metabolism.

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Riboswitch RNAs: Sensing Metabolic Signals with RNA Transcripts

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With 1 Figure

Abstract

Direct sensing of an effector molecule by a nascent RNA transcript has emerged as a common mechanism for regulation of gene expression in bacteria. RNAs of this type, termed "riboswitches," respond specifically to the cognate regulatory signal. This interaction modulates the structure of the nascent transcript, which can determine whether the RNA folds into the helix of an intrinsic terminator, resulting in premature termination of transcription. Similar RNA rearrangements mediate translational regulation by sequestration of the ribosome binding site. Each class of riboswitch RNA recognizes its signal with high specificity and an affinity appropriate to the *in vivo* pools of the effector. Characterization of the RNA-effector interaction in these systems has provided new information about how different classes of signals are recognized, and about the impact of these regulatory mechanisms on the cell.

Zusammenfassung

Die direkte Erkennung eines Effektormoleküls bei RNA-Transkripten *in statu nascendi* (isn) wurde erst spät als allgemeiner Mechanismus der Regulation der Genexpression bei Bakterien anerkannt. Dieser RNA-Typ, der als „riboswitch" bezeichnet wird, reagiert spezifisch auf das entsprechende Regulatorsignal/-molekül. Das Signalmolekül verändert die Sekundärstruktur des isn-Transkripts dergestalt, dass die RNA eine Terminatorstruktur ausbildet, was zur vorzeitigen Beendigung der Gentranskription führt. Reife m-RNA kann durch Signalmoleküle Strukturen ausbilden, die die Ribosomenbindungsstelle markieren und damit zur translationalen Regulation führen. Jeder „riboswitch"-RNA-Typ wird hochspezifisch von seinem entsprechenden Signalmolekül erkannt, und seine Bindungsaffinität ist auf die typischen Konzentrationen der Signalmoleküle/Effektoren in der Zelle abgestimmt. Die genaue Charakterisierung der RNA-Effektor-Wechselwirkung hat zu neuen Informationen über den Erkennungsmechanismus der verschiedenen Effektorklassen und ihre Bedeutung im Regulationsmechanismus der Zelle geführt.

1. Introduction

Early studies of bacterial gene expression focused on regulation at the level of transcription initiation using DNA binding proteins to modulate the interaction of RNA polymerase (RNAP) with the promoter region of the target gene to either activate or repress promoter activity. Analyses of the *Escherichia coli trp* and *Salmonella typhimurium his* operons, and the bacteriophage lambda developmental program, revealed that regulation could also occur after RNAP has left the promoter region through placement of a transcription termination site in the region between the transcription start-site and the beginning of the regulated coding sequence(s) (Henkin and Yanofsky 2002). This region of the gene is designated the leader region and the corresponding region of the transcript is designated the leader RNA.

A termination signal within the leader region is called an attenuator, and results in two possible transcription products: (*i*) a short terminated RNA product that does not include the regulated coding sequences; or (*ii*) a full-length RNA transcript that includes the coding sequences. Synthesis of the full-length RNA is necessary for expression of the downstream genes, allowing regulation of gene expression by regulation of the activity of the leader region terminator.

Transcription termination in bacteria most commonly uses intrinsic terminators, which are comprised of a short G+C-rich helix immediately followed by several consecutive U residues in the RNA transcript. A rarer class of terminators relies on the Rho protein factor. Most genes regulated by transcription attenuation use intrinsic terminators, and the activity of terminators of this type can be controlled by the ability of the leader RNA to fold into an alternate antiterminator structure that competes with the terminator helix by sequestration of residues that form the 5' side of the terminator helix. Regulation is therefore dependent on modulation of the structure of the nascent RNA to determine whether the nascent transcript folds into the terminator helix (resulting in no transcription of the downstream region) or the competing antiterminator helix (resulting in readthrough of the termination site and synthesis of the full-length transcript).

The earliest transcription termination control systems that were discovered utilize either the translation of a leader peptide coding sequence or the binding of an RNA-binding protein to modulate the structure of the nascent RNA transcript (Grundy and Henkin 2006). These types of systems use either the translating ribosome or a RNA-binding protein to monitor an appropriate physiological signal (e.g., abundance of a particular aminoacyl-tRNA or a small molecule that affects the RNA-binding activity of the regulatory protein either directly or indirectly). More recently, regulatory systems have been discovered in which the signal is monitored directly by the nascent RNA transcript, without the assistance of a ribosome or regulatory protein. Systems of this type, termed riboswitches, have been shown to regulate a wide variety of genes in bacteria and to operate not only at the level of transcription attenuation, but also at the level of translation initiation by using similar RNA structural rearrangements to determine whether the RNA transcript contains an accessible ribosome-binding site or instead folds into a structure in which the ribosome-binding site is sequestered into a competing RNA element (Henkin 2008).

2. RNA-dependent Riboswitches: the T Box Mechanism

The T box mechanism was first discovered through analysis of the *Bacillus subtilis tyrS* gene, which encodes tyrosyl-tRNA synthetase (Henkin et al. 1992). Regulation was shown to occur at the level of transcription attenuation, and expression is induced by conditions that result in accumulation of uncharged tyrosyl-tRNA. Phylogenetic studies showed that features identified in the *tyrS* leader RNA are conserved in a large number of amino acid-related genes, especially in Firmicutes, including a variety of genes encoding aminoacyl-tRNA synthetases, amino acid biosynthetic enzymes, and amino acid transporters (Grundy and Henkin 1993, Gutierrez-Preciado et al. 2009).

A triplet sequence embedded at a conserved position within the leader RNA structure was found to correspond to a codon whose amino acid specificity matches the amino acid specificity of the downstream regulated coding sequence (Grundy and Henkin 1993). For example,

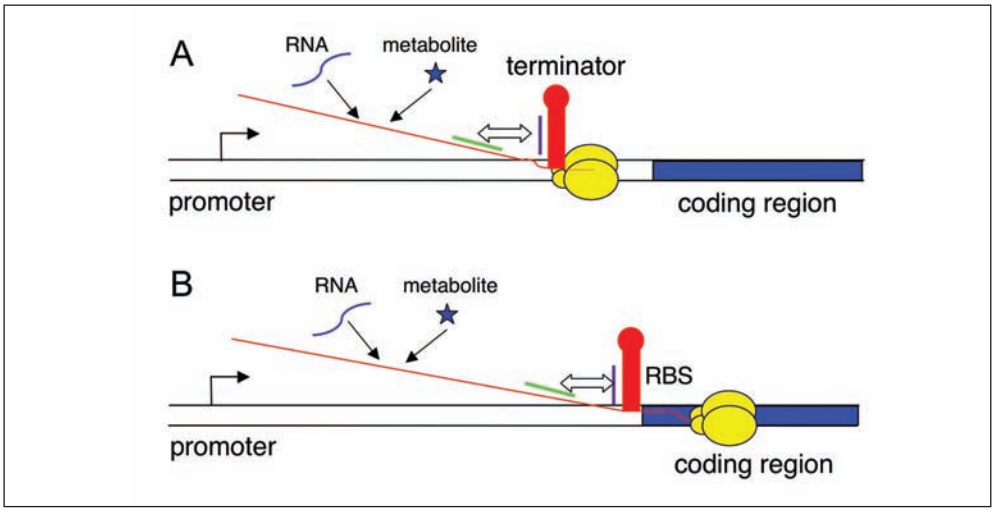

Fig. 1 Riboswitch-mediated regulation of gene expression. (*A*) Transcription attenuation control. As RNAP (yellow ovals) proceeds along the DNA (double black line), the nascent leader RNA (red line) can fold into the helix of an intrinsic terminator (red stem-loop). This results in premature termination of transcription, and RNAP fails to transcribe the downstream coding region (blue rectangle). Pairing of sequences on the 5' side of the terminator (purple line) with complementary sequences further upstream (green line) results in the formation of a competing antiterminator structure. The binding of an effector molecule (RNA or metabolite) changes the RNA structure and determines whether the leader RNA folds into the terminator or antiterminator structures. (*B*) Translational control. RNAP transcribes through the leader region and into the downstream coding region. The RNA can fold into a structure (red stem-loop) that sequesters the ribosome-binding site (RBS), resulting in inhibition of translation initiation. The pairing of sequences on the 5' side of the structure that sequesters the SD (purple line) with complementary sequences further upstream (green line) results in the formation of a competing structure that allows binding of the translation initiation complex to the RBS. The binding of an effector molecule (RNA or metabolite) changes the RNA structure and determines whether the leader RNA folds into the terminator or antiterminator structures.

the *tyrS* gene has a UAC tyrosine codon, *pheS* has a UUC phenylalanine codon, *trpS* has a UGG tryptophan codon, etc. Genetic analyses demonstrated that this codon, designated the Specifier Sequence, directs pairing with the anticodon of the cognate tRNA, and is therefore responsible for the specificity of the regulatory response of each gene in the T box family. Replacement of the *tyrS* UAC with a UAG nonsense codon results in loss of expression; this phenotype is suppressed by the introduction of the matching nonsense suppressor tRNA into the cell, providing clear demonstration that tRNA is the effector (Grundy and Henkin 1993).

Genetic studies also demonstrated that the unpaired residues at the acceptor end of uncharged tRNA (NCCA) pair with four residues (UGGN) in the antiterminator bulge (Grundy et al. 1994). These studies showed that uncharged tRNA promotes antitermination, and that the cognate charged tRNA acts as an inhibitor of antitermination, presumably by binding to the Specifier Sequence and blocking access of uncharged tRNA. This led to a basic model in which each T box family leader RNA senses the ratio of charged and uncharged forms of a specific tRNA species, to determine whether RNAP will terminate or transcribe through the termination site and synthesize the full-length transcript.

The *in vivo* genetic studies, while clearly showing the role of tRNA, could not provide information about whether tRNA is sufficient to promote antitermination. An *in vitro* transcrip-

tion system was therefore used to test for the ability of tRNA to promote readthrough of the leader region terminator in the absence of other cellular factors. These studies demonstrated that the nascent leader RNA can discriminate between cognate and noncognate tRNA, and that tRNA-dependent antitermination can occur in a purified system (Grundy et al. 2002).

Further biochemical analyses showed that purified leader RNA can form a complex with the cognate uncharged tRNA, allowing mapping of structural changes in both RNA partners (Yousef et al. 2005). These studies not only confirmed the Specifier Sequence-anticodon and antiterminator-tRNA acceptor end interactions, but also revealed structural changes at other regions of both RNAs. The nature of these other structural changes, and the corresponding leader RNA-tRNA interactions, remain to be identified.

3. Metabolite-dependent Riboswitches

Recent studies have revealed a large number of gene families in which regulation occurs by the direct binding of a small molecule to the leader RNA (Henkin 2008). The binding of the regulatory molecule results in a structural rearrangement of the RNA that can affect transcription termination or translation initiation, thereby controlling expression of the downstream coding sequences. Two different classes of riboswitch RNAs that monitor *S*-adenosylmethionine (SAM) will be described as examples.

3.1 SAM-dependent Transcription Termination: the S Box Riboswitch

Examination of a number of genes involved in biosynthesis of methionine and SAM in *B. subtilis* revealed the presence of a conserved leader RNA pattern, designated the S box (Grundy and Henkin 1998). Genetic and physiological studies demonstrated that expression of these genes is low under conditions when intracellular SAM pools are high and expression is induced when SAM pools are reduced (Grundy and Henkin 1998, Tomsic et al. 2008). This regulatory response is dependent on direct binding of SAM to the leader RNA which promotes activity of the leader region terminator (McDaniel et al. 2003). SAM is specific, as related compounds, including *S*-adenosylhomocysteine (SAH), do not bind. Structural studies revealed that SAM is completely surrounded by the RNA in the SAM-bound structure (Montange and Batey 2006) and SAM binding promotes formation of the terminator helix by the sequestration of sequences that would otherwise form a competing antiterminator, as predicted by the model (Grundy and Henkin 1998, McDaniel et al. 2003).

The S box mechanism is widely used to regulate methionine-related genes in *Bacillus* and *Clostridium* sp. In contrast, it is found only sporadically in the *Lactobacillales.* In organisms that use the S box mechanism for any gene, the *metK* gene, encoding SAM synthetase, is nearly always preceded by an S box element. This raised the question of how *metK* is regulated in members of the *Lactobacillales* that lack the S box mechanism.

3.2 SAM-dependent Translational Control: the S_{MK} Box Riboswitch

The majority of *metK* genes from members of the *Lactobacillales* (including *Enterococcus, Streptococcus* and *Lactobacillus* sp.) were found to contain a similar leader RNA pattern,

designated the S_{MK} box (Fuchs et al. 2006). This RNA pattern includes two alternate forms, one of which was predicted to sequester a portion of the Shine-Dalgarno (SD) sequence by pairing with a complementary anti-Shine-Dalgarno (ASD) sequence; sequestration of the SD was predicted to inhibit translation initiation and, since *metK* is involved in the synthesis of SAM, we proposed that the binding of SAM to the RNA would promote SD-ASD pairing and reduced SAM synthetase production.

The S_{MK} box RNA from *Enterococcus faecalis* was shown to bind SAM *in vitro*, and genetic studies showed that disruption of the SD-ASD pairing blocks binding of SAM. Binding is specific, as SAH had no activity, and structural mapping revealed that the SD is exposed in the absence of SAM, and protected in the SAM-RNA complex (Fuchs et al. 2006). Binding of SAM to the RNA inhibits binding of 30S ribosomal subunits (Fuchs et al. 2007), consistent with the model. Crystal structure analysis of the S_{MK} box RNA-SAM complex revealed that SAM sits in a pocket formed by the SD-ASD pairing, and directly contacts residues in the SD sequence (Lu et al. 2008).

The recognition of SAM is completely different in the S box and S_{MK} box riboswitches, and follows yet another pattern in a third SAM-binding riboswitch (Gilbert et al. 2008). These studies demonstrate the high versatility of RNA in recognition of (and discrimination between) complex biological molecules, as three different RNA-based strategies to utilize SAM as a regulatory molecule have evolved in different bacterial lineages.

4. Conclusions and Perspectives

The ability of RNA to serve as a regulatory molecule has only recently been recognized. RNAs can act in trans to regulate expression of target genes, as in the case of the small RNAs, or as tRNA acts in the T box mechanism. RNAs can also act in cis to directly recognize regulatory signals and regulate downstream gene expression, as in the riboswitches and RNA thermosensors. Riboswitch RNAs are by definition colocalized with their regulatory targets allowing for easy movement as a unit from organism to organism by horizontal gene transfer. This ability to be transferred readily to new genetic backgrounds, and the fact that the riboswitch directly recognizes a signal that is conserved in the new background (i.e., a tRNA or a normal cellular metabolite), is likely to be responsible for the widespread presence of these elements in a variety of bacterial lineages. The observation that certain mechanisms are found in certain groups of bacteria is probably a function both of evolutionary history and physiological constraints specific to that lineage.

The rapid progress in identification of riboswitch elements, and structural characterization of RNA-ligand complexes, provides significant new information about the ability of RNA to serve as a regulatory molecule. Open questions include how each riboswitch confers specific ligand recognition, and the basis for variability in affinity of naturally occurring riboswitch variants for the same ligand (Tomsic et al. 2008). In addition, since most of the structural information available is limited to the RNA-ligand complex, there is little information about the free form of the RNA, and the ligand-induced transitions to the bound form. Finally, while it is clear that many riboswitch RNAs can function in the absence of cellular factors, it remains possible that currently unknown factors participate in riboswitch function *in vivo*.

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Global Regulation of Bacterial Metabolism

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Role of the Second Messenger Cyclic-di-GMP in the Coordination of Growth Phases, the General Stress Response and Biofilm Formation of *Escherichia coli*

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With 1 Figure

Abstract

During entry into stationary phase, initially flagellated motile single *E. coli* cells loose their motility and produce (auto)adhesive curli fimbriae and extracellular matrix compounds that allow them to form surface-associated multicellular communities, i.e. biofilms. A small signaling molecule, c-di-GMP, plays a crucial role in this "life-style" switch. C-di-GMP is produced and degraded by diguanylate cyclases (carrying GGDEF domains) and specific phosphodiesterases (EAL domains), respectively, many of which are under the control of σ^S (RpoS). From the molecular characterization of several of these systems, which in *E. coli* regulate switching from motility to adhesion as well as the composition and architecture of biofilms, a novel concept for the mechanisms and evolution of second messenger signaling has recently emerged.

Zusammenfassung

Beim Eintritt in die stationäre Phase verlieren zunächst flagellierte motile *E.coli*-Einzelzellen ihre Beweglichkeit und produzieren (auto)adhäsive Curli-Fimbrien und extrazelluläre Matrixkomponenten, mit deren Hilfe sie oberflächenassoziierte multizelluläre Gemeinschaften, d.h. Biofilme, bilden. Ein kleines Signalmolekül, c-di-GMP, kontrolliert diesen Übergang zwischen verschiedenen „Lebensstilen". C-di-GMP wird von Diguanylatcyclasen (mit GGDEF-Domänen) synthetisiert und von spezifischen Phosphodiesterasen (mit EAL-Domänen) abgebaut. Die Expression vieler dieser Enzyme wird von σ^S (RpoS) aktiviert. Aus der molekularen Charakterisierung einiger dieser Systeme, die in *E. coli* das Umschalten von Motilität zu Adhäsion sowie Zusammensetzung und Architektur von Biofilmen regulieren, ergibt sich nunmehr ein neues Konzept zu Mechanismen und Evolution der Signalübertragung durch *Second-messenger*-Moleküle.

1. Bacterial "Life-style" and the Ubiquitous Signaling Molecule Cyclic-di-GMP

Most bacteria can occur as motile single cells in the 'planktonic' state, but can also switch to the sessile and usually multi-cellular life-style that is characteristic for biofilms. Biofilm formation occurs in several stages (Stoodley et al. 2002). These include a down-regulation of motility and induction of various surface adhesins, which allow cell-cell aggregation and attachment to abiotic or biotic surfaces, followed by the formation of microcolonies, secretion of matrix exopolysaccharides and further proliferation that can result in complex morphological structures. Within such structures, bacteria are highly resistant against antibiotics, disinfectants, and the attacks of the immune systems of host organisms. Biofilm formation is involved in chronic and medical device-related infections, and biofilms in the environment

represent underestimated reservoirs for the dissemination of pathogenic bacteria (Anderson and O'Toole 2008, Hall-Stoodley and Stoodley 2005).

In general, biofilm formation is promoted by the ubiquitous nucleotide second messenger bis-(3'-5')-cyclic di-guanosine-mono-phosphate (c-di-GMP). c-di-GMP also inhibits various forms of motility, i.e. it controls switching between the motile-planktonic and sedentary biofilm-associated life-styles of bacteria (for recent reviews, see Hengge 2009, 2010, Jenal and Malone 2006). Regulation by c-di-GMP requires (*i*) controlled production and degradation of this second messenger, (*ii*) effector components that sense c-di-GMP by directly binding it, and (*iii*) targets, whose output activity is controlled by direct interaction with the effectors. c-di-GMP is synthesized by diguanylate cyclases (DGC) characterized by the GGDEF domain (named after the conserved amino acid motif representing the active centre), which often also have a secondary c-di-GMP binding site ('I-site') for allosteric product feed-back inhibition. Degradation of c-di-GMP is mediated by specific phosphodiesterases (PDE), which can feature either EAL or HD-GYP domains. Many of these enzymes, some of which are membrane-bound, also have various N-terminal sensory input domains that control their activities in response to intra- and extracellular signals. C-di-GMP-binding effectors can be proteins or RNAs (riboswitches). Targets controlled by these effectors are highly diverse, including promoter regions, RNAs, enzymes or complex molecular structures such as the flagellar basal body or exopolysaccharide synthesis and excretion machineries.

The striking multiplicity of DGCs and PDEs (especially in proteobacteria[1]) may provide the basis to link different DGCs and PDEs to different outputs in parallel-operating pathways and thereby greatly enhance the flexibility of c-di-GMP signaling. These considerations have lead to the concept of *sequestration* of c-di-GMP control modules (Hengge 2009). Sequestration means that not all c-di-GMP control modules (i.e. a set of DGC, PDE, effector and direct target that together produce a distinct output) should be present and active at the same time and place. Sequestration can be *temporal*, i.e. by differentially regulating cellular levels and activities of the components of such modules in response to intra- or extracellular signals that change over time. In addition, sequestration may be *functional*, i.e. entire c-di-GMP control modules (with different regulatory outputs) could operate at the same time, but physically separated from each other. Functional sequestration implies local action and therefore most likely direct interaction of the DGCs, PDEs, effector and target components involved; this may result in temporarily higher local concentrations of c-di-GMP or even the generation of local c-di-GMP pools due to some structural confinement (forming a 'microcompartment' or 'microdomain').

2. Inverse Coordination of Motility and Curli-mediated Adhesion in *E. coli* Integrates Global and Local c-di-GMP Signaling

In *E. coli*, c-di-GMP signaling and the initiation of biofilm formation, i.e. essentially the decision to "swim or stick", is intimately connected to the regulatory circuitry that controls the transition from post-exponential to stationary phase and the general stress response: The main components of this circuitry are the (*i*) flagellar control cascade directed by the master

1 For instance, most *E. coli* strains have 29 GGDEF/EAL domain proteins (Hengge 2010, Sommerfeldt et al. 2009).

regulator FlhDC, (*ii*) the stationary phase and general stress response coordinated by the σ^S (RpoS) subunit of RNA polymerase (RNAP), and (*iii*) various biofilm functions controlled by the regulatory protein CsgD, which is itself under multiple σ^S control (Fig. 1).

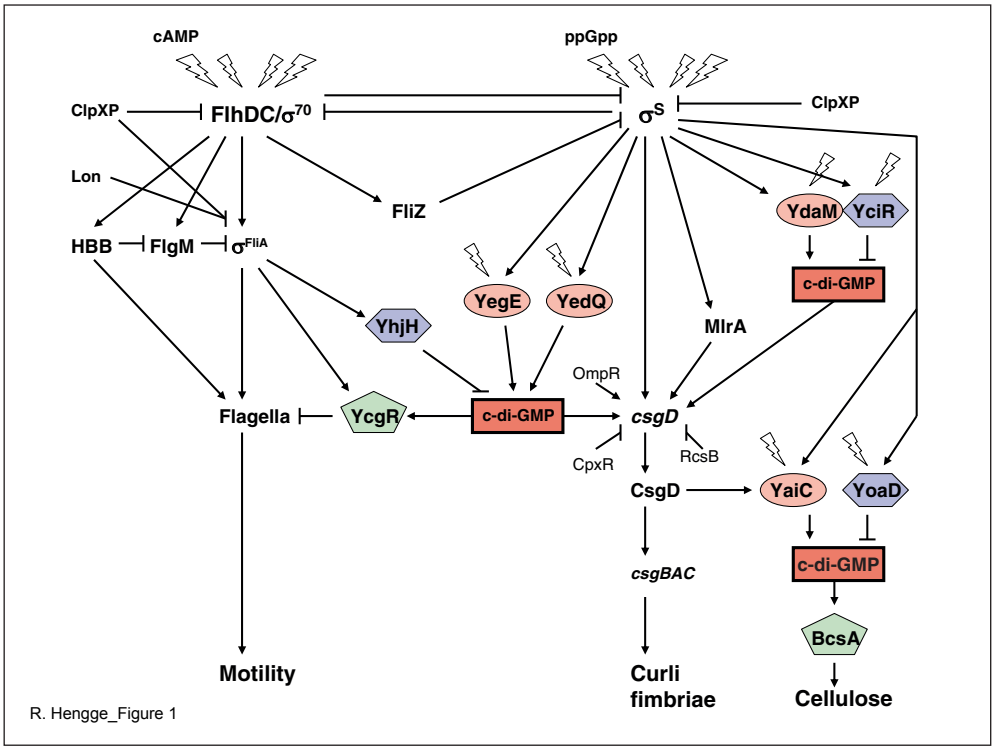

Fig. 1 c-di-GMP control modules involved in the inverse coordination of motility and synthesis of curli fimbriae and cellulose in *Escherichia coli*. Diguanylate cyclases, phosphodiesterases and effectors, that have been shown to bind c-di-GMP, are indicated by light red, blue and green symbols, respectively. For detailed explanation and references, see main text. This figure is a modified version of a figure published by Hengge (2010), used here with permission.

The post-exponential growth phase, where resources are limited but not yet exhausted, is a foraging phase, in which *E. coli* cells become highly motile (Adler and Templeton 1967, Amsler et al. 1993, Pesavento et al. 2008). In this phase, a PDE under FlhDC control (YhjH) keeps the cellular c-di-GMP level low, which is a prerequisite for flagellar activity. However, if this strategy is not successful, i.e. when resources dwindle further and growth slows down, motility is reduced again and adhesins such as the autoaggregative curli fimbriae are expressed, i.e. cells prepare for biofilm formation, which later (after surface attachment) leads to the production and secretion of matrix exopolysaccharides (e.g. cellulose). This phase is characterized by a strong induction of σ^S and the general stress response, which includes induction of DGCs (YegE, YedQ), which outbalance the PDE activity of YhjH, such that c-di-GMP accumulates. As a result, cells switch from motility to adhesion, i.e. they reduce

flagellar activity (via the c-di-GMP-binding effector YcgR) and concomitantly induce CsgD, an activator essential for the expression of curli fimbriae and cellulose (Fig. 1) (Pesavento et al. 2008). In addition, a second c-di-GMP control module (involving the DGC YdaM, the PDE YciR) and the transcription factor MlrA are essential for CsgD/curli induction (Brown et al. 2001, Weber et al. 2006). This second system seems a prototype of a locally acting module, since all three components interact (S. Lindenberg and R. Hengge, unpublished data) and this system acts exclusively in CsgD/curli control but does not 'cross-talk' into motility control (Pesavento et al. 2008, Sommerfeldt et al. 2009). Once expressed, CsgD also induces the expression of the DGC YaiC, which together with the PDE YoaD, is part of a c-di-GMP control module that lateron induces the production of cellulose (Fig. 1) (Brombacher et al. 2006). As strong CsgD induction occurs only below 30 °C (Römling 2005), these processes play a major role in biofilm formation in the environment.

3. Evolution of c-di-GMP Signaling: the Emergence of Alternative Functions for GGDEF/EAL Domain Proteins

Functional sequestration and local operation of c-di-GMP control modules based on direct protein interactions can open new pathways for the evolution of signal transduction: enzymatic activities of the DGC and/or PDE may get lost, with signal transfer now using the former substrates or products as allosteric ligands for controlling the activities of interacting components or even relying exclusively on macromolecular interactions. This provides a rationale for the existence of 'degenerate' GGDEF/EAL domain proteins, in which amino acids crucial for DGC or PDE activity are not conserved, and therefore cannot function by producing or degrading c-di-GMP.

A system recently characterized at the molecular level (Tschowri et al. 2009) involves the *E. coli* proteins YcgF, a degenerate EAL domain protein with a blue light-sensing N-terminal BLUF domain, and YcgE, a paralog of the curli-controlling transcription factor MlrA. YcgF does not degrade nor bind c-di-GMP, but instead directly interacts with YcgE and, in a blue light-controlled manner, releases YcgE from its operator sites. As a result a distinct regulon is induced, which includes the *ycgZ-ymgABC* operon. Strikingly, most regulon members are small proteins (below 100 amino acids). Among these, YmgB (and to a minor extent,YmgA) controls a number of biofilm-associated functions via the RcsC/RcsD/RcsB two-component phosphorelay pathway. These include a stimulation of colanic acid production and a down-regulation of CsgD, and therefore curli and cellulose production. Thus, the YcgF/YcgE/YmgB/Rcs pathway seems involved in shaping biofilm composition and architecture, indicating that despite its loss of c-di-GMP signaling, this system remains within the same physiological context as the 'canonical' c-di-GMP signaling systems.

4. Conclusions and Perspectives

From the recent studies on the molecular mechanisms of c-di-GMP signaling, a concept emerges which distinguishes three classes of signaling systems that involve GGDEF/EAL domain proteins: (*i*) 'classical' second messenger systems, in which c-di-GMP is freely diffusible, and the cellular level of c-di-GMP can be balanced by several DGCs and PDEs (that

are expressed depending on the specific conditions); (*ii*) locally acting second messenger systems, in which the local c-di-GMP concentration is varied depending on the activities of a DGC and a PDE that interact in a complex which also includes the effector and the direct target; and finally, (*iii*) signaling systems involving 'degenerate' GGDEF/EAL domain proteins that have 'given up' c-di-GMP signaling, that in some cases may still use GTP or c-di-GMP as allosteric ligands and that now directly rely on macromolecular interactions for signal transfer (Hengge 2009, Pesavento and Hengge 2009). A prerequisite for the emergence and co-existence of these systems in a single bacterial species is the multiplicity of DGCs and PDEs. In the light of this novel concept of second messenger signaling, it is tempting to speculate that in certain alpha-proteobacteria and mycobacteria, which (unlike enteric bacteria) feature multiple adenylate cyclases (Galperin 2005, Shenoy et al. 2004), cAMP signaling may have undergone a similar diversification.

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Coordination of Metabolism and Gene Regulation by Trigger Enzymes

Jörg Stülke and Fabian M. Commichau (Göttingen)

With 2 Figures

Abstract

To respond to changes in the environment, bacteria possess a large set of regulatory systems. These systems are often composed of dedicated sensing and regulatory modules. For sensing the availability of metabolites, enzymes are the components of the cell that have this information at their direct disposal. This corresponds well with the information that many enzymes have 'moonlighting' activities in signal transduction. Collectively, these enzymes are called trigger enzymes. They affect gene expression by their ability to directly bind DNA or RNA targets in response to the availability of their metabolites or cofactors. Moreover, trigger enzymes may act by controlling the activity of transcription factors, either by protein phosphorylation or by regulatory protein-protein interactions. Finally, some glycolytic enzymes participate in the control of mRNA degradation. Thus, enzymes play an important role not only as catalysts, but also in the control of gene expression and metabolic fluxes.

Zusammenfassung

Um auf Veränderungen der Umweltbedingungen reagieren zu können, besitzen Bakterien sehr viele Regulationssysteme. Diese Systeme sind oft aus Modulen für die Signalwahrnehmung und die Reaktion aufgebaut. Um die Verfügbarkeit von Metaboliten wahrnehmen zu können, sind Enzyme am besten geeignet, da ihnen diese Information unmittelbar zur Verfügung steht. Solche Enzyme, die neben ihrer katalytischen eine regulatorische Aktivität haben, werden als Triggerenzyme bezeichnet. Sie kontrollieren die Genexpression entweder durch direkte Bindung an bestimmte DNA- oder RNA-Sequenzen oder durch die Modulation der Aktivität von Transkriptionsfaktoren, entweder durch Phosphorylierung oder durch regulatorische Protein-Protein-Interaktionen. Schließlich sind einige glykolytische Enzyme an der Kontrolle des mRNA-Abbaus beteiligt. Damit spielen Enzyme auch eine wichtige Rolle bei der Kontrolle der Genexpression und der Stoffwechselflüsse.

1. Introduction

To be successful in competition with their fellows, all organisms need to detect changes in their environment and respond to them rapidly and appropriately while preventing the waste of energy and resources. In bacteria, the main level at which these regulatory events occur is transcription. Therefore, several mechanisms allowing tight control of transcription have evolved.

In each case, regulatory events require the perception of the environmental or internal signal and its transduction to the transcription machinery. Many transcription factors integrate signal perception and transcription regulation in a single molecule, among them the Lac repressor or the cAMP receptor protein from *Escherichia coli*. In many other cases, signal rec-

ognition and the regulatory output are embodied in two distinct proteins, subunits or domains that interact with each other for signal transduction. Two-component regulatory systems are the paradigm of this type of regulation (Laub and Goulian 2007). The separation of the two tasks in signal transduction is very advantageous in evolution as it allows shifts in specificity by gene duplication and specialization.

There are proteins in the cell that are well informed about specific conditions, usually about metabolite availabilities. These proteins are the enzymes that recognize their substrates and that often undergo structural alterations upon interaction with the substrate or during their reaction. This makes the enzymes perfect candidates to share this information with transcription regulators, or to regulate gene expression themselves. Such proteins that exert a second, unrelated function, in addition to their primary task, are called "moonlighting proteins" (Jeffery 1999). A plethora of enzymes involved in transcription regulation from a variety of biochemical pathways has recently been discovered, suggesting that this regulatory principle is much more common than hitherto acknowledged. These enzymes are collectively designated as trigger enzymes to emphasize their role in signal transduction. Trigger enzymes can exert different functions. They might act as transcription factors by direct binding to either DNA or RNA, or they might modulate the activity of transcription factors either by covalent modification or by protein-protein interactions (Commichau and Stülke 2008).

2. Trigger Enzymes Active as DNA-binding Transcription Factors

In bacteria, transcription is usually controlled by DNA-binding repressor or activator proteins. The recognition of a specific sequence requires the presence of a protein domain that is able to recognize and bind a DNA motif. Since DNA recognition motifs are usually not present in enzymes, the acquisition of such a domain is a prerequisite for the evolution of an enzyme to a DNA-binding trigger enzyme.

In *E. coli* and other enterobacteria, a bifunctional enzyme catalyzes the two-step degradation of proline to glutamate. This enzyme, PutA, is also involved in transcription regulation of the divergent *putA* and *putP* genes encoding the bifunctional proline utilization protein and the proline transporter, respectively (Ostrovsky de Spicer and Maloy 1993). This regulation is dependent on the availability of proline.

PutA can bind directly to five conserved sites in the promoter region of the *putA* and *putP* genes, thus causing transcriptional repression in the absence of proline (Zhou et al. 2008). On the other hand, the enzymatically active, membrane-bound form of PutA is unable to bind DNA and to repress transcription (Ostrovsky de Spicer and Maloy 1993). Several studies have addressed the identity of the molecular inducer that causes release of PutA from the DNA, resulting in transcription of the *put* genes. In contrast to many other repressors, two factors contribute to induction: the presence of proline and FAD induces a conformational change in PutA that results in the shuttling of the protein from the DNA to the membrane (Zhu and Becker 2003).

PutA contains two large domains: The N-terminal domain has the proline dehydrogenase (PDH) activity whereas the C-terminal domain harbors the Δ^1-pyrroline-5-carboxylate dehydrogenase activity. The conformational change that occurs in the presence of proline and FAD was mapped to the PDH domain (Zhu and Becker 2003). The DNA-binding activity of PutA

is located in the N-terminal 47 amino acids of the PDH domain that form a ribbon-helix-helix (RHH) motif (Gu et al. 2004).

Similar to PutA, the trigger enzymes BirA and NadR from *E. coli* possess distinct DNA-binding domains. Interestingly, BirA seems to act as a transcription factor in many bacteria and even in some archaea (Rodionov et al. 2002).

3. Trigger Enzymes Involved in Post-transcriptional Regulation via Protein-RNA Interaction

RNAs can adopt a variety of structures, and they can bind and interact with virtually any molecule, including metabolites and proteins. Thus, it is not surprising that proteins with very different structures are able to bind RNA and, indeed, many enzymes interact with RNAs and exert regulatory effects by this interaction.

There is so far only one example of a bacterial trigger enzyme with RNA-binding activity, aconitase. Aconitase catalyzes the reversible conversion of citrate to isocitrate in the tricarboxylic acid (TCA) cycle and requires an iron-sulphur cluster for activity (Volz 2008). Under conditions of iron-limitation, the TCA cycle cannot operate due to the inactivity of aconitase. However, aconitase can help solve this problem by binding so-called iron-responsive elements (IREs) in the mRNAs of genes involved in iron homeostasis. This was first shown for the human enzyme, but later also for the aconitases from such diverse bacteria as *E. coli*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* (Commichau and Stülke 2008).

The determination of the structure of IRE-bound aconitase revealed the molecular basis for the two mutually exclusive activities. In the presence of iron, the protein has a compact conformation. In the absence of iron, the iron-sulphur cluster disassembles and the free (apo-) aconitase adopts a more open conformation. This opening allows the binding of the IRE. It is worth noting that only a few bases are conserved in the iron-responsive elements, and that these conserved bases are brought into the right position by secondary structure elements (Walden et al. 2006).

Based on the overall sequence similarity of the eukaryotic and *B. subtilis* aconitases and IREs (Alén and Sonenshein 1999), it seems safe to assume that the *B. subtilis* enzyme follows the same mechanism as outlined above for the mammalian enzyme. *B. subtilis* aconitase binds IREs in the untranslated regions of the *qoxD* and *feuAB* mRNAs. These genes encode the iron-containing protein cytochrome aa_3 oxidase and an iron uptake system, respectively (Alén and Sonenshein 1999).

E. coli possesses two aconitases, of which aconitase B (AcnB) is the main enzyme involved in the TCA cycle. In contrast to the monomeric enzymes of eukaryotes and *B. subtilis*, AcnB has a dimerization domain. An analysis of the requirements of AcnB for RNA binding revealed that it is independent of the iron-sulphur cluster in the active centre of the enzyme. Moreover, the arrangement of the RNA in the protein seems to be different from that observed in the mammalian aconitase-IRE complex (Tang et al. 2005).

There is a large variety of potential RNA structures that might be able to bind diverse ligands and, indeed, many eukaryotic enzymes have been shown to moonlight in RNA interactions. Thus, it would not be surprising if many more bacterial enzymes turn out to be trigger enzymes involved in protein-RNA interactions.

4. Trigger Enzymes Controlling Gene Expression by Signal-dependent Phosphorylation of Transcription Regulators

In many bacteria, sugars are transported by the phosphoenolpyruvate phosphotransferase system (PTS). The PTS permeases are not only involved in sugar transport; they also control the activity of transcription activators and antiterminators by phosphorylating them in response to the availability of the respective substrate. These transcription regulators all share a duplicated, so-called PTS regulation domain (PRD) (Stülke et al. 1998). Phosphorylation of one of these domains by the sugar-specific permease occurs in the absence of the substrate (upon accumulation of phosphorylated permease) and results in the inactivation of the regulators. In the presence of the substrate, the phosphate groups are drained to the sugar and the regulators become dephosphorylated and, thus, regain activity. This regulatory mechanism has been most intensively studied for the control of β-glucoside transport in *E. coli* and for glucose and fructose uptake in *B. subtilis* (Amster-Choder and Wright 1990, Görke 2003, Martin-Verstraete et al. 1998, Schmalisch et al. 2003).

5. Trigger Enzymes Controlling the Activity of Transcription Factors by Protein-Protein Interactions

The fourth class of trigger enzymes controls gene expression by modulating the activity of transcription factors – either activators or repressors. The diversity of such interactions reflects the diversity of transcription factors and makes it difficult to predict these trigger enzymes from the primary sequence.

Trigger enzyme activity is not restricted to PTS permeases. Recently, the *E. coli* lysine transporter LysP was shown to control the activity of the transcription activator protein CadC in response to the availability of lysine (Tetsch et al. 2008). Interestingly, CadC is a membrane protein with a cytoplasmic DNA-binding domain. It has therefore been hypothesized that CadC itself might sense the lysine concentration. In a series of excellent biochemical and genetic experiments, Tetsch et al. (2008) provide compelling evidence that CadC is unable to bind lysine, and that the signal is instead sensed by the lysine transporter LysP and transduced to CadC via interaction of the trans-membrane domains of the two proteins. In the absence of lysine, LysP is thought to bind and sequester CadC, thus inhibing its DNA-binding activity. If lysine is present, then LysP is involved in transporting this amino acid, and CadC becomes free and can now activate the expression of its target operon, *cadBA* (Tetsch et al. 2008).

In *B. subtilis*, a trigger enzyme controls glutamate biosynthesis. In this bacterium, the glutamate dehydrogenase RocG inhibits the activity of the transcription factor GltC in the presence of arginine (see Fig. 1). RocG participates in arginine degradation and is induced in the presence of arginine. The enzyme catalyzes the last step of the pathway, the conversion of glutamate to 2-oxoglutarate. Thus, arginine utilization results in the formation of glutamate and circumvents the need for glutamate biosynthesis by the glutamate synthase, the product of the *gltAB* operon. This is achieved by a regulatory interaction between RocG and the transcription activator of the *gltAB* operon, GltC (Commichau et al. 2007). The expression of the *rocG* gene is not only subject to induction by arginine but also to glucose repression. In consequence, complete repression of *rocG* by glucose also allows activity of free GltC, and thus expression of the *gltAB* operon and glutamate biosynthesis. This regulatory mechanism

couples ammonium assimilation and anabolic reactions to the availability of carbon sources and the flux through glycolysis that results in carbon catabolite repression (COMMICHAU et al. 2006).

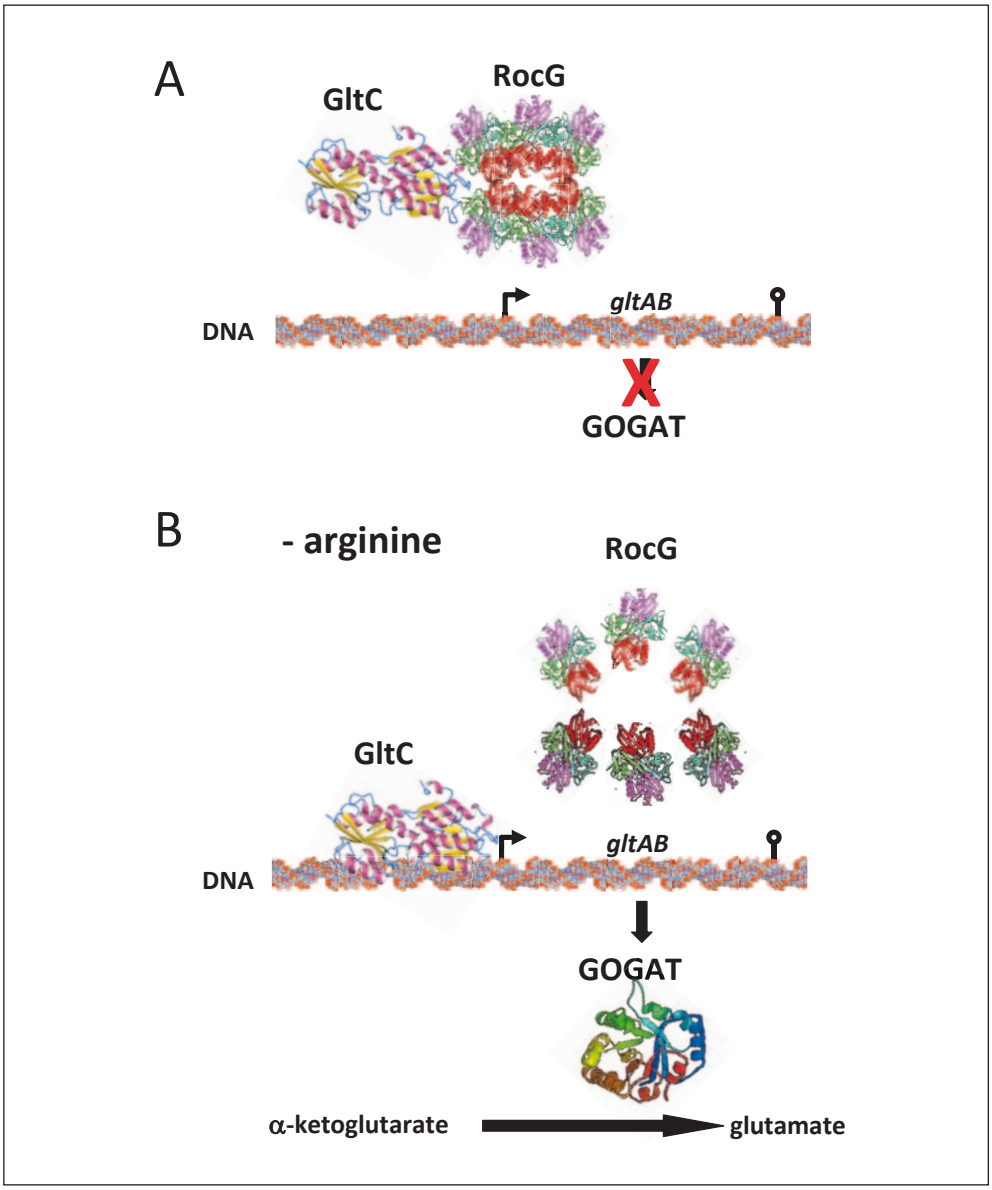

Fig. 1. Control of glutamate biosynthesis in *B. subtilis* by the trigger enzyme RocG. (*A*) In the presence of arginine, the glutamate dehydrogenase catalyzes the oxidation of glutamate to 2-oxoglutarate. The enzymatically active protein interacts with the transcription factor GltC and prevents it from activating the transcription of the *gltAB* operon encoding glutamate synthase. (*B*) In the absence of arginine, no active RocG is available to inactivate GltC. Under these conditions, GltC binds its DNA target and activates transcription eventually resulting in the synthesis of glutamate.

6. Moonlighting Enzymes Involved in mRNA Processing and Degradation

In both *E. coli* and *B. subtilis*, glycolytic enzymes are part of a protein complex involved in mRNA degradation and processing, the RNA degradosome. In *E. coli*, the degradosome is a large multi-protein complex that consists of the major endoribonuclease RNase E, the polynucleotide phosphorylase, the ATP-dependent RNA helicase RhlB, and enolase (Carpousis 2007, see Fig. 2).

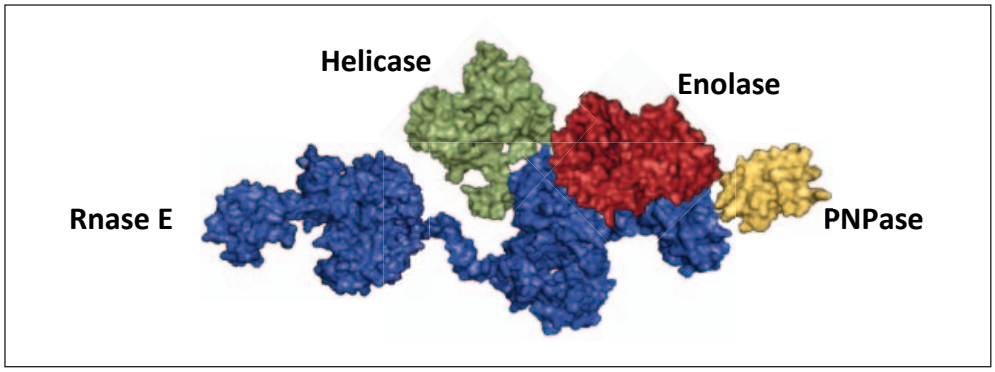

Fig. 2 The RNA degradosome of *E. coli*. The RNA degradosome is a multiprotein complex that consists of endo- and expribonucleases (RNase E and polynucleotide phosphorylase PNPase, respectively), a RNA helicase and the glycolytic enzyme enolase.

The role of enolase within the complex has been enigmatic until recently. This enzyme plays a crucial role in the regulation of the *ptsG* mRNA (encoding the major glucose transporter) stability in response to metabolic stress. Thus, glycolytic activity might be linked to RNA metabolism by a regulatory protein-protein interaction *via* enolase (Morita et al. 2004). A recent analysis of interaction partners of glycolytic enzymes in *B. subtilis* revealed that enolase and phosphofructokinase are part of the RNA degradosome in this organism. Interestingly, this degradosome consists of three RNases, polynucleotide phosphorylase, and a RNA helicase (Commichau et al. 2009). The role of glycolytic enzymes in this complex remains to be elucidated.

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Medicine at the Interface between Science and Ethics

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Naturwissenschaft und Theologie/Ethik versuchen mit unterschiedlichen Konzepten, ein Weltbild zu erfassen, das die *conditio humana* besser zu verstehen erlaubt. Die Fragen sind weit gefasst; endgültige Antworten wird man nicht leicht finden. Gemeinsame Diskussionen über diese Probleme könnten beiden Gebieten Anregungen geben und der Biomedizin im Umgang mit der sehr kritischen Öffentlichkeit helfen. Voraussetzung ist Offenheit gegenüber der anderen Denkweise. Der vorliegende Band behandelt daher aus der Perspektive von Naturwissenschaftlern und Ethikern so verschiedene Themen wie die neuen Herausforderungen an Moral- und Ethikdiskurse durch die jüngsten Fortschritte der Biowissenschaften, die Grenzen der ethischen Reflexion bei den neueren Entwicklungen der Molekularbiologie, die Geschichte der Auffassungen vom „Gen“ und seiner Bedeutung in der Humanbiologie, aber auch die Missverständnisse zwischen den beiden Kulturen der Naturwissenschaften und der Geisteswissenschaften in der Forschung über Lebensprozesse. Dazu kommen Beiträge zur Stammzellproblematik, der Verwendung von Tiermodellen in der Translationsmedizin, über Würde von Zellen in Kultur, Fragen der Pluripotenz von Zellen und der Reprogrammierung von Zellkernen sowie der Bedeutung von Methylierungsmustern für die Epigenetik. Die Beiträge sind in englischer oder deutscher Sprache verfasst.

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Metabolomics Systems Biology – a New Tool for Virulence Research

Rainer Breitling (Groningen), Richard A. Scheltema (Groningen), and Michael P. Barrett (Glasgow)

With 4 Figures

Abstract

Metabolomics systems biology aims at understanding the dynamic metabolome of an organism. A new generation of mass spectrometers, capable of mass separation of complex mixtures at very high resolution and mass accuracy, supports this approach by offering the possibility of rapidly exploring uncharted territories of the metabolic map. Ultra-accurate pre-processing achieves mass accuracies better than 0.5 ppm; accurate masses allow robust metabolite identification; and this can be exploited for the reliable *ab initio* inference of metabolic pathways. Metabolomics systems biology offers exciting opportunities for virulence research. With the help of new bioinformatics tools, it will provide a uniquely detailed view of microbial metabolic pathways in action.

Zusammenfassung

Metabolom-orientierte Systembiologie hat zum Ziel, das dynamische Metabolom eines Organismus zu verstehen. Eine neue Generation von Massenspektrometern unterstützt diesen Ansatz: sie ermöglicht einen schnellen Überblick über große unerforschte Stoffwechselregionen mit extremer Massenauflösung und -genauigkeit auch für komplexe Mischungen. Durch ultra-akkurate Aufbereitung der Daten können Massengenauigkeiten von unter 0.5 ppm erreicht werden; die genaue Masse erlaubt die robuste Identifizierung der Moleküle; und diese kann verwendet werden, um zuverlässig neue Stoffwechselwege zu rekonstruieren. Metabolom-orientierte Systembiologie bietet interessante Möglichkeiten für die Virulenzforschung. Mit Hilfe von neuen bioinformatischen Methoden verschafft sie einen einmalig detaillierten Einblick in den mikrobiellen Stoffwechsel in Aktion.

1. Introduction

Metabolomics, the comprehensive measurement of the metabolite content in biological samples, has been a late addition to the toolbox of post-genomic biology (Fiehn 2002) and holds great promise for future studies of metabolic effects on bacterial virulence. The technical challenges in metabolite analysis are much more complex than those encountered in gene expression studies (such as microarray experiments) and even in proteomics (the global study of all cellular proteins). The chemical and biophysical diversity of metabolites is substantially greater than that of proteins or nucleic acids, which makes comprehensive analysis, preferentially in a single experiment, a daunting task.

Despite these challenges, several forms of metabolomics have been among the major success stories of systems biology (Kell 2004). By the 1970s, the quantitative study of metabolic networks was one of the driving forces for the establishment of a more integrative,

systems-oriented approach to molecular biology. Methods such as metabolic control analysis showed the power of quantitative approaches by generating highly counterintuitive insights into the function of metabolic systems (Fell 1992). In the post-genomic era, metabolomics systems biology has been most prominently represented by constraint-based genome-wide models of metabolic networks (Reed et al. 2006). Using sequence homology to identify all potential enzymes encoded by a genome, one creates a global stoichiometric matrix for a metabolic network, which is then analyzed by methods such as flux balance analysis to predict metabolic fluxes and mutant phenotypes. This approach has been applied to a variety of bacterial pathogens (for example, *Salmonella typhimurium* [Raghunathan et al. 2009], *Porphyromonas gingivalis* [Mazumdar et al. 2009], *Neisseria meningitidis* [Baart et al. 2007, 2008], *Pseudomonas* spec. [Oberhardt et al. 2008, Puchalka et al. 2008], *Mycobacterium tuberculosis* [Raman et al. 2005], *Helicobacter pylori* [Schilling et al. 2002]).

Genome-wide stoichiometric modeling has been very successful, but it has limitations. Perhaps most importantly, it necessarily focuses on those reactions that are well studied, either in the target organism or (more commonly) in any of the major model species. As a result, the models tend to be most accurate for central metabolism, which is also the major target of quantitative approaches to metabolomics systems biology, such as dynamic modeling using ordinary differential equations. This is a serious restriction, as a large amount of the most interesting metabolomic diversity is generated at the fringes of the metabolome (Fig. 1). These compounds – which include pheromones, colorants, toxins, and many others – tend to be involved in interactions with the organic environment, for instance between host and pathogen or between microbes within a biofilm. This, as a general rule, favors diversifying selection, whether by molecular arms races or by favoring selective communication channels. Such taxon-specific molecules, produced by equally taxon-specific pathways, will often be invisible to genome-wide models based on sequence homology. To overcome this focus on the core metabolome, which potentially overemphasizes the similarity between organisms, it is necessary to continue developing new approaches for experimentally exploring the secondary metabolome, as comprehensively as possible.

One important bottleneck of such a comprehensive metabolomics approach, aiming at novel compounds, is the identification of metabolites, once they have been separated and quantified. The reasons for the difficulty of identifying metabolites are quite obvious: one cannot "sequence" a metabolome in the same way as a genome, using a single technological platform. Even obtaining a comprehensive metabolic profile is a vastly more difficult task given the chemical diversity of the compounds studied and range in concentrations of metabolites (van der Werf et al. 2007). Furthermore, there is no simple genetic code that tells us how metabolites are connected to enzymes. Consequently, many metabolomics studies have been restricted to generating metabolite fingerprints or diagnostic profiles without identifying the majority of detected compounds, but for a systems biological approach such anonymous information is not sufficient.

Here we discuss one approach to exploring the "blind spot" of metabolism by a combination of liquid chromatography and high-resolution mass spectrometry. The presentation focuses on data from metabolomics pilot experiments using the Orbitrap mass analyzer, a member of a new generation of mass spectrometers, capable of mass separation of complex mixtures at very high resolution and mass accuracy (Breitling et al. 2006a).

Due to their high resolution, these new mass spectrometers generate an abundance of metabolome data. Novel bioinformatics solutions are needed to fully utilize this information.

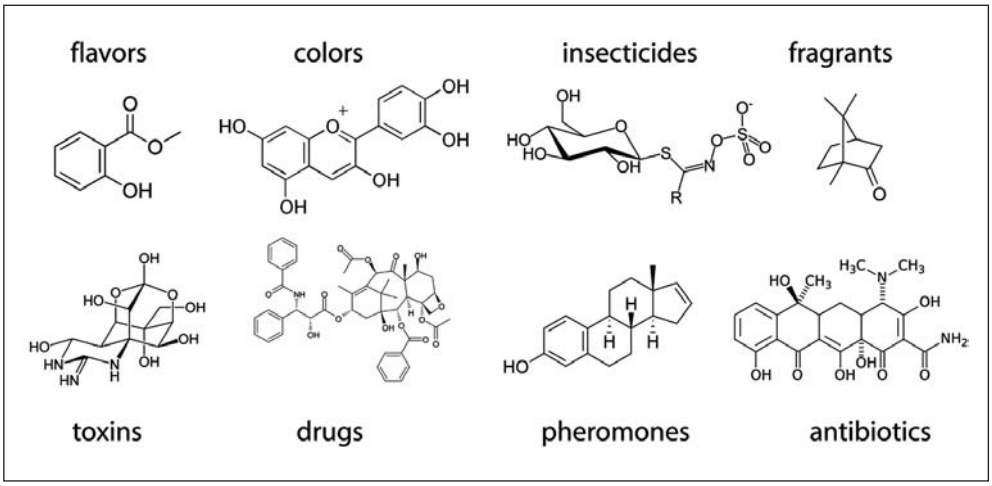

Fig. 1 Secondary metabolites at the fringes of the metabolome. These chemically and functionally highly diverse compounds illustrate the abundance of biotechnologically and biomedically relevant small biomolecules that are the target of metabolomics approaches. Shown are methyl salicylate ("oil of wintergreen", a flavorant involved in anti-herbivore defence), cyanidin (a red-blue antioxidant anthocyanidin found in red berries, apples, plums, cabbage), a glucosinolate (natural pesticides in many plants of the cabbage family), camphor (a strong aromatic, used for cooking and embalming), tetrodotoxin (a neurotoxin blocking voltage-gated channels in nerves, responsible for pufferfish poisoning), taxol (paclitaxel, one of the major anti-cancer drugs), estratetraenol (a potential pheromone in humans), and tetracycline (a broad-spectrum polyketide antibiotic produced by *Streptomyces rimosus*).

We discuss some of the algorithms we have recently developed for that purpose and show that they offer the possibility of exploring uncharted territories on the metabolic map at an unprecedented rate.

2. Accuracy-Based Pre-Processing

Two features of the first Orbitrap mass spectra we analyzed (Fig. 2) were the enormous amount of information and the ubiquity of background ions, which are detected in all spectra and almost all chromatographic fractions. Initially, we decided to remove these signals, because the standard analysis software was reporting results that consisted almost entirely of ubiquitous contaminants rather than the cellular metabolites that were the target of our studies. Due to the high resolution of the data, detection and removal of ubiquitous masses is straightforward (although a computationally efficient algorithm had to be developed to perform the spectral cleaning rapidly on spectra of several hundred megabytes in size). However, it turned out that the background ions could also be exploited for precise spectral alignment of the mass-over-charge axis across multiple samples. Furthermore, using the high level of accuracy of the equipment, we were able to identify many of the contaminants by matching them to lists of known background ions in the chemical literature and confirming the identification by tandem mass spectrometry. Identified background ions covered most of the mass range of interest. As we then knew the exact molecular mass, we could compare it to the observed mass and thus use the contaminants for calibration. Their ubiquity meant that their observed mass was esti-

mated with a high degree of precision, and we were consequently able to achieve an unprecedented reproducible average mass accuracy of less than 0.5 ppm (Scheltema et al. 2008).

3. Chemical Formula and Structure Prediction

Once the mass of metabolites is estimated with sufficient accuracy, the exact mass can be used for metabolite identification. An accuracy of better than 0.5 ppm means that usually only a single atomic composition will match the observed mass within the confidence limits (Kind and Fiehn 2006). Of course, isomers will not be distinguished (a general limitation of mass spectrometry), but the identification of the correct atomic composition can already serve as a powerful guide for successful database searches. When metabolites are not yet recorded in the database, it is possible to exhaustively enumerate possible atomic compositions (typically

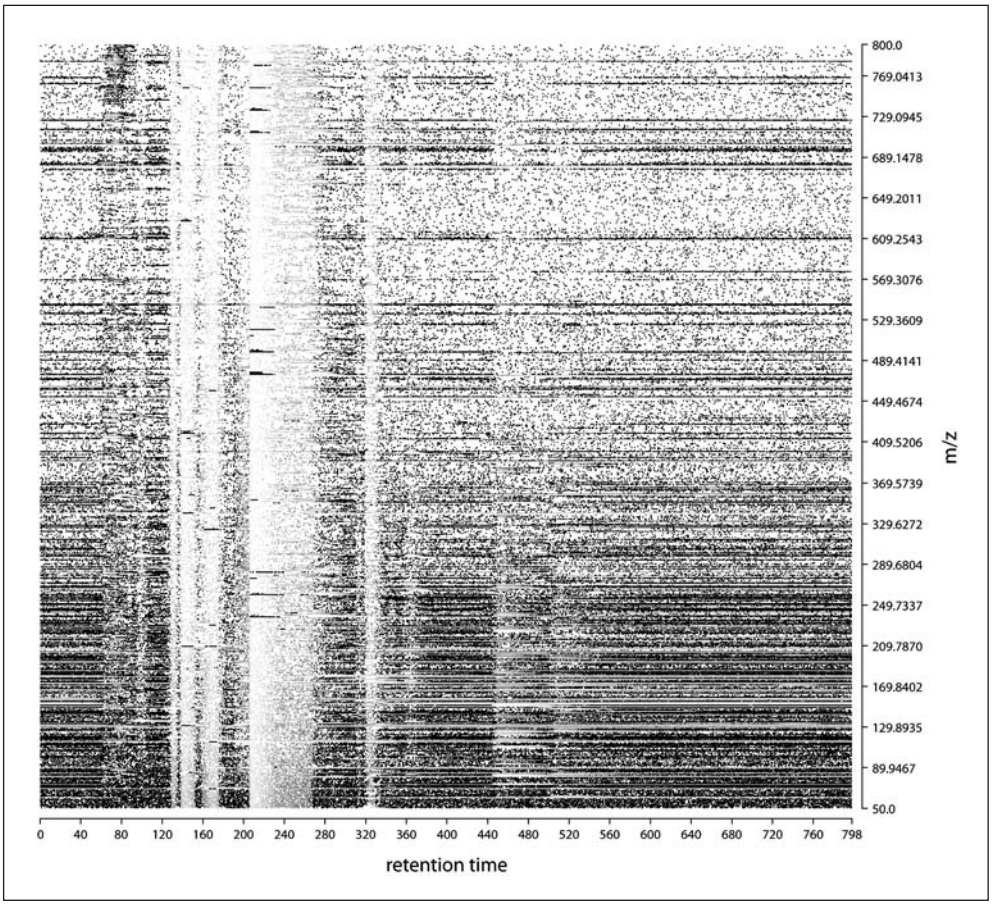

Fig. 2 Example of a high-accuracy mass spectrum of a metabolome sample. The x-axis corresponds to chromatographic retention time, the y-axis to mass-over-charge ratio, and the coloring indicates the intensity of the signal (black, lowest; white, highest). Background ions are visible as dark horizontal bands.

limited to carbon, hydrogen, nitrogen, oxygen, sulfur and phosphate) and still find a unique match. This match can then be examined for likely structural motifs that would match the predicted composition. In addition, isotope pattern matching can be used to exclude spurious formulas, taking into account the differences in natural isotope abundance for different elements (Kind and Fiehn 2007). Identified metabolites are easy to match to metabolic networks, providing informative visualizations of the data (Fig. 3).

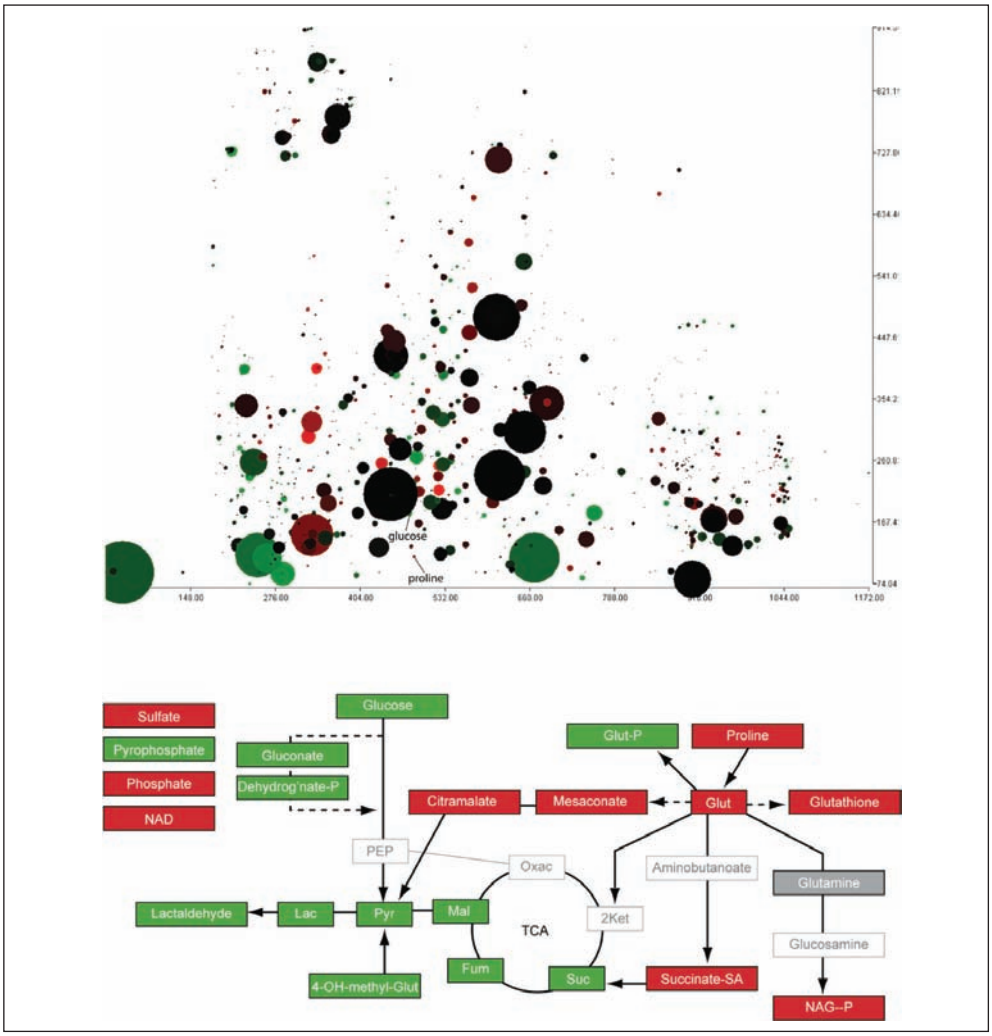

Fig. 3 Mass-based metabolite identification and matching to metabolic networks. The plot on top shows metabolites identified in a high-accuracy mass spectrum after alignment and accurate calibration. The axes are the same as in Figure 2. The size of the circles corresponds to the maximum signal detected, the color to the relative signal in two growth conditions (green: up-regulated in glucose-fed cells; red: up-regulated in proline-fed cells). The picture below shows the most strongly changed metabolites projected onto a map of the metabolic network using the same color coding. Metabolites in white were not detected. This mapping provides a powerful visualization for exploring the metabolic context of observed changes (modified with permission from Scheltema et al. 2008).

4. *Ab Initio* Network Reconstruction

High mass accuracy also implies highly accurate mass differences between metabolically linked metabolites. Every metabolite in the sample should be related by some kind of biochemical transformation to other metabolites that, in the ideal case, would also be observed in the same spectrum. These transformations usually come from a limited set of options (oxidations, dehydrogenations, phosphorylations, etc.), each of them corresponding to a characteristic mass difference (Breitling et al. 2006b). This can be exploited in multiple ways to create a systematic overview of the metabolome. First, it is possible to use the metabolomic context of observed masses to decide among alternative formulas or structures. This is most important for low-intensity ions, which have less accurate mass estimates: the presence of potential metabolic precursors can strengthen the preference for identification as a specific formula or structure. We have implemented this concept in an integrated statistical framework that identifies the most likely assignment of empirical formulas for an entire dataset (Rogers et al. 2009). Second, the inferred transformations between measured metabolites can be used to construct metabolome-wide networks *ab initio* (Fig. 4). These can be linked to existing network maps and are an important step towards expanding the scope of metabolomics systems biology from the core metabolome to the fringes of secondary metabolism. This approach is currently applied to studies of drug resistance mechanisms and virulence in the protozoan pathogen *Leishmania donovani* (Decuypere, Scheltema et al. unpublished results).

The MetabolomeExplorer software that serves as an integrated test bed for these approaches is currently in the beta-testing phase and being prepared for public release (Scheltema et al. in preparation). It will help experimental biologists explore the rich data created by new mass spectrometers by automatically performing network reconstruction, formula searches and *ab initio* prediction of atomic composition (and potentially structural motifs).

5. Conclusions and Outlook

Metabolomics systems biology using ultra-high resolution mass spectrometry is a rapidly emerging field. To achieve its full potential, it will be necessary to combine mass spectrometry data with a variety of orthogonal sources of evidence (Breitling et al. 2008). For example, studying time-resolved stable-isotope labeling patterns will prune predicted networks of spurious pathways: if an isotope label does not pass from metabolite A to metabolite B, any hypothetical pathway between the two is clearly not relevant. Performing systematic perturbation experiments to determine how a perturbation spreads throughout the metabolic network is an additional important and very general strategy for deciding which metabolites are in fact connected. Genetical genomics, a multifactorial perturbation approach, is particularly promising, because it also provides a direct link to the genome. A recent pilot study in *Arabidopsis* (Fu et al. 2009) has integrated genetical genomics data based on metabolite screens, proteomics and gene expression, with classical phenotype information, to provide a first global overview of molecular robustness in the cellular system. In the future, the approach will also become a powerful tool for studying the mechanisms of microbial virulence. It is already becoming clear that changes in metabolism play crucial roles in microbial virulence. Early studies into virulence using metabolomics have shown, for example, that malaria parasites deplete arginine within their locality, possibly to reduce the availability of this substrate for the host to

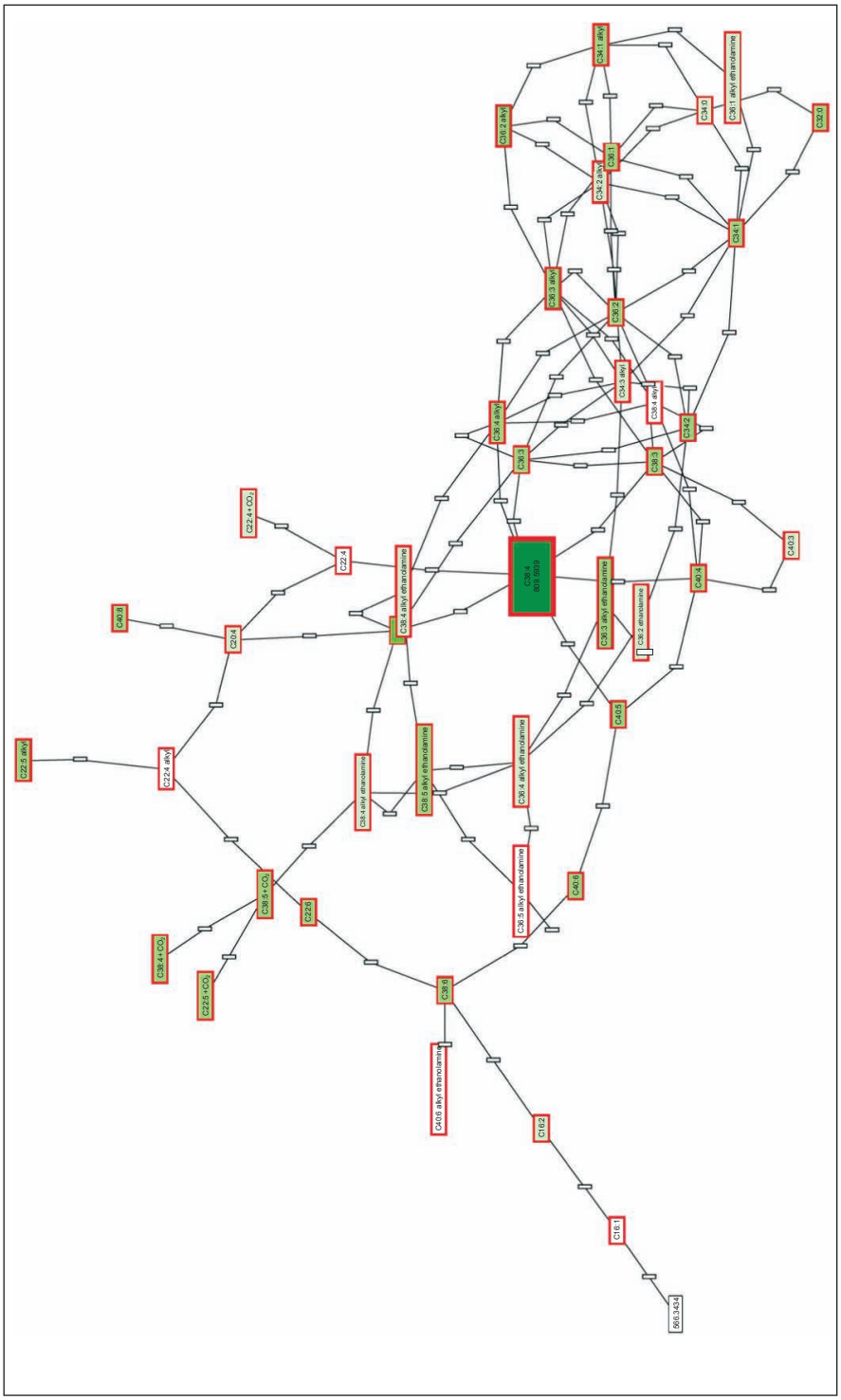

Fig. 4 Metabolite network inferred *ab initio* from high-accuracy metabolomics data in *Trypanosoma brucei* (BREITLING et al. 2006b). Nodes correspond to observed metabolites and are colored according to the correlation of metabolite abundance in a panel of experimental conditions, relative to the central metabolite (dark green). Only a sub-network of correlated metabolites is shown.

generate defensive nitric oxide (Olszewski et al. 2009). Changes to fatty acid metabolism in infected cells appear to be critical to virulence of human cytomegalovirus (Munger et al. 2008). It is well known that assemblies of low molecular weight metabolites are critical virulence factors in bacterial cell walls, and other low molecular weight, non-proteinaceous, secretion products play key roles as toxins. With the help of new bioinformatics tools, metabolomics systems biology will provide a uniquely detailed view of metabolic pathways in action.

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Individuelle und globale Ernährungssituation – gibt es (noch) eine Lösung?

Gemeinsames Symposium
der Deutschen Akademie der Naturforscher Leopoldina
und der Österreichischen Akademie der Wissenschaften (ÖAW)

vom 30. bis 31. Oktober 2008 in Wien

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Der Anteil der Menschen, die übergewichtig bis fettleibig sind, ist heute ebenso groß wie der Anteil derjenigen, die an Hunger leiden – jeweils fast eine Milliarde! Die Beiträge hinterfragen, welche Handlungsalternativen wir im Hinblick auf unsere individuelle und globale Ernährungssituation haben. Sie zeigen auf, was wir tun können, um den globalen Hunger zu bekämpfen, und was wir tun müssen, um die individuelle Ernährungssituation in den Griff zu bekommen.
Zurzeit sehen wir mit der Umnutzung von Ackerböden für den *Non-Food*-Bereich Entwicklungen, die die globale Nahrungsmittelverfügbarkeit weiter verschärfen. Dazu werden Fragen der Nahrungsmittelproduktion und Verteilung – bis hin zum Einsatz von Gentechnik – behandelt, um eine ausreichende Versorgung der Weltbevölkerung zu erreichen. Der Band diskutiert Ursachen und Folgen von Über- und Mangelernährung und hebt besonders die gesundheitspolitische Relevanz der Ernährungsfrage hervor.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Metabolic Adaptations of Pathogens with Extracellular or Intracellular Life Style

Nova Acta Leopoldina NF *111*, Nr. 378, 85 – 93 (2010)

Metabolic State: The Key Regulator of Virulence in *Staphylococcus aureus*

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Abstract

Historically, metabolism and virulence have been treated as distinct areas in the field of microbiology. However, recent studies demonstrate that metabolism and virulence are linked; hence, they must be considered together if one is to understand how bacteria coordinate these activities. The discovery that small colony variants (SCVs) of *Staphylococcus aureus* have decreased electron transport activity and toxin production led to studies examining how metabolism and expression of pathogenicity factors are intertwined (Proctor et al. 2006). An early and striking observation was that SCVs completely lacked RNAIII (Vaudaux et al. 2002), a riboregulator involved in multiple facets of pathogenesis in staphylococci. The complete loss of RNAIII is striking because *S. aureus* has a baseline level of RNAIII at all times, suggesting that the absence of RNAIII was an active process, *i.e.*, repression, degradation, instability, etc., mediated through the metabolic state of the organism. Subsequent to this observation, metabolism has been shown to influence/control the activity of over 15 regulators involved in the biosynthesis of virulence factors. Thus, to understand and characterize virulence in *S. aureus*, one must always consider and control for the metabolic state of the organism.

Zusammenfassung

Historisch gesehen hat sich die Erforschung des bakteriellen Metabolismus und der Pathogenität zunächst getrennt und unabhängig voneinander entwickelt. Jedoch ist inzwischen klar, dass Metabolismus und Pathogenität regulatorisch vernetzt sind und dass die Koordination dieser Vernetzung verstanden werden muss. Die Entdeckung, dass „small colony variants" (SCVs) von *Staphylococcus aureus* ein vermindertes elektrochemisches Membranpotential haben, was mit einer verminderten Toxinproduktion assoziiert ist, lässt vermuten, dass die Aktivität des Metabolismus die Produktion von Pathogenitätsfaktoren beeinflussen kann (Proctor et al. 2006). Schon frühzeitig fiel auf, dass SCVs keine RNAIII haben (Vaudaux et al. 2002), die als Riboregulator vielfältig die Pathogenität von Staphylokokken koordinieren kann. Die komplette RNAIII ist normalerweise in größeren oder kleineren Mengen in Staphylokokken vorhanden und verschwindet nur völlig bei SCVs. Dies weist auf aktive Prozesse wie Transkriptionsrepression, RNA-Degradation u. a. hin, die vom metabolischen Zustand der Zelle kontrolliert werden. Inzwischen konnte gezeigt werden, dass über den metabolischen Zustand der Zelle die Aktivität von über 15 Regulatoren, die an der Produktionssteuerung von Pathogenitätsfaktoren beteiligt sind, kontrolliert wird. Deshalb erfordert die Analyse der Pathogenität die Einbeziehung von metabolischen Vorgängen.

1. Introduction

The title of this manuscript is intentionally provocative so as to emphasize the point that the metabolic state has a very important function in the expression of virulence factors. When one compares the past two decades of literature with the preceding four decades, one can appreciate that the emphasis has changed dramatically from an examination of growth conditions

that influenced the expression of virulence factors, to a search for regulatory molecules. The watershed event came with the discovery in Richard Novick's laboratory that there was an accessory gene regulator (Agr) that regulated the synthesis of α-toxin (Recsei et al. 1986). This was followed about a decade later by the identification of the staphylococcal accessory regulator (Sar) (Cheung and Projan 1994, Cheung et al. 1997a), the repressor of toxins (Rot) (McNamara 2000), and a many other regulators, including several Sar homologues (Cheung et al. 2008). Identification of these regulators revealed a complex network of interacting regulators, most of which are negative regulators (Arvidson and Tegmark 2001). Indeed, *S. aureus* regulation is best characterized as a hierarchy of negative regulators that negatively regulate other regulators; thus, positive regulation occurs when there is negative regulation of a negative regulator. Despite a thorough understanding of virulence regulation, there remained the major unanswered question of why metabolic events have such a profound effect on the regulation of toxin production?

The answer to this question traces back to some of the earliest studies of toxin production, where it was recognized that oxygen tension had profound effects on the production of hemolysins (Coleman 1985). Similarly, studies of *S. aureus* isolates involved in the tampon-associate, toxic shock epidemic revealed that microaerophilic conditions and carbon dioxide levels had a major effect on toxic shock syndrome toxin (TSST-1) production (Ross and Onderdonk 2000). In addition to oxygen tension, it has been observed that glucose and unregulated pH suppressed toxin synthesis (Regassa et al. 1991, 1992). Not only do environmental/nutritional conditions influence toxin synthesis, but the production of toxins depends on the phase of growth, leading to the hypothesis that a temporal factor increased secreted toxin synthesis late in the exponential phase of growth (Vandenesch 1991). More recently, studies of electron transport defective *S. aureus* variants have revealed profound decreases in secreted toxin production and increases in many cell-associated adhesins (Proctor et al. 2006). These electron transport defective strains provided several insights into the relationship between metabolism and virulence factor regulation. These strains had a distinctive phenotype: Slow growth on agar plates produced small, non-hemolytic colonies that did not ferment mannitol and had very slow coagulase production, but did have increased antibiotic resistance (Proctor et al. 2006). Clinically, SCVs were able to cause persistent, recurrent, and antibiotic-resistant infections (Proctor et al. 1995), which has been associated with the ability of SCVs to persist within host cells (Proctor et al. 1994, Sendi and Proctor 2009 Vesga et al. 1996). The characterization of SCVs and the increased interest in how metabolism influences the regulation of virulence factor production is reviewed herein.

Basics of toxin regulation: Agr is a quorum sensing system encoded within the *agr* locus and consists of two divergently transcribed loci controlled by two promoters, P2 and P3, that regulate transcription of a two-component regulator and a regulatory RNA known as RNAIII (Novick et al. 1995). Four genes are controlled by P2: *agrA*, *agrC*, *agrD*, and *agrB*. AgrC is the transmembrane histidine kinase component of the *agr* two-component regulatory system, with AgrA being the response-regulator. AgrD is a small peptide that is processed by AgrB into a cyclic thiolactone peptide also known as the auto-inducing peptide (AIP) (Ji et al. 1997, Mayville et al. 1999). As the extracellular concentration of AIP increases, the likelihood that it will encounter and complex with AgrC increases, resulting in the activation of the AgrC kinase domain (Lina et al. 1998). AgrC phosphorylates AgrA, which increases transcription from the P2 and P3 promoters (Koenig et al. 2004). Transcription from P3 produces the untranslated riboregulator RNAIII and the mRNA for δ-toxin encoded therein (Janzon et al.

1989, Novick et al. 1993). RNAIII enhances the synthesis of secreted virulence determinants and represses the synthesis of surface associated proteins (*e.g.*, protein A and fibronectin binding protein) (Recsei et al. 1986). RNAIII regulates through its action as an antisense RNA that binds to *agr* regulated mRNAs, such as *hla* (α-toxin) and *spa* (protein A) (Benito et al. 2000, Boisset et al. 2007, Huntzinger et al. 2005, Morfeldt et al. 1995), which results in degradation of RNAIII-mRNA complexes by directing the activity of endoribonuclease III (RNase III) (Huntzinger et al. 2005).

As mentioned earlier, staphylococci often achieve positive regulation through negative regulation of negative regulators. In that vein, SarA is a negative regulator of the *agr* operon (Cheung et al. 2008). Since the discovery of *sarA*, a number of SarA homologues have been described that form a complex regulatory scheme. Except for SarU, these regulators are repressors (Arvidson and Tegmark 2001, Cheung et al. 2008). A detailed review of this regulatory cascade is available (Cheung et al. 2008), but an example of how these Sar homologues relate to metabolism is provided here. The stress sigma factor, σ^B (SigB), is important for the activation of the *sarA* operon (Cheung et al. 1999). σ^B is hypothesized to respond to environmental signals and is involved in SarA activation. SarA activates Agr, which represses SarT. SarT is an activator of SarS, which is a repressor of *hla* (α-toxin) and an activator of *spa* (protein A). Similarly, Rot responds to environmental signals as well, and it represses *hla*, probably through SarT and SarS interactions.

2. Effects of Metabolic State on Regulators

SigB: σ^B is hypothesized to respond to stresses, including acid and metabolic stress (Bischoff 2001, Cheung et al. 1999, Kullik and Giachino 1997). Activation of the σ^B regulatory cascade involves a series of anti- and anti-anti-sigma factors that free σ^B so that it can act on Sar A and other σ^B-dependent promoters. In turn, SarA regulates a number of regulators involved in virulence factor production via Agr (*e.g.*, α-toxin, enterotoxins, TSST-1, proteases), and also biofilm formation (Cheung et al. 1994, 1997a, b, 1999, 2008, Karlsson-Kanth 2006, Knobloch et al. 2001, Palma and Cheung 2006, Schmidt et al. 2004, Xiong et al. 2006). Taken together, σ^B links toxin production to a bacterial general stress response.

Catabolite repressors: In a wide variety of bacteria, glucose is known to repress a number of pathways, and this is known as catabolite repression. *S. aureus* has a typical response to glucose via CcpA (Deutscher et al. 1994, 1995, Galinier et al. 1997, Wray et al. 1994). Glucose is known to repress RNAIII and toxin synthesis in *S. aureus* (Regasa et al. 1991, 1992), and more recent data supports the concept that this occurs via CcpA (Seidl et al. 2008). Glucose also suppresses the expression of capsular polysaccharides; however, it stimulates the production of protein adhesins (Seidl et al. 2008). While many of these responses to glucose can be related to RNAIII, the situation is more complex as the target sequence for CcpA is a *cre* site, and this is not present in the promoters of *agr* and capsule genes, but is found before *hla* (α-toxin), *tst* (toxic shock syndrome toxin), and *spa* (protein A) (Somerville and Proctor 2009).

GTP-dependent regulation: CodY is a transcriptional repressor that responds to the intracellular concentrations of GTP and branched chain amino acids (BCAA) (Shivers and Sonenshein 2004). CodY's affinity for its DNA-binding site is increased by binding, but not hydrolyzing, GTP and this affinity is increased further by binding a BCAA. In *S. aureus*, CodY regulates

protease production and biofilm formation (Somerville and Proctor 2009); thus, CodY links the intracellular concentration of the metabolic products GTP and BCAA to virulence.

ATP-dependent proteases: Casinolytic proteases (Clp) bind ATP and sense stress when ATP is depleted (Frees et al. 2005). Clps are two-component proteases with an ATP-binding protein (*e.g.*, ClpC) that can act as a chaperone for certain proteins under ATP-rich conditions and a protease component, such as ClpP (Frees et al. 2007). As ATP levels decline, ClpC disassociates from ClpP, thereby allowing it to become proteolytically active. Of interest, the target for the protease is often the protein that ClpC has been chaperoning. This has relevance to *S. aureus* virulence factor regulation when one considers that Rot is a target of ClpX-ClpP. Thus, falling ATP levels result in digestion of Rot and increased expression of toxins (Frees et al. 2005). ClpC is also needed for aconitase function, which influences toxin production via TCA cycle activity (discussed below) (Chatterjee et al. 2005, 2007, 2009).

Nitrogen-dependent regulator: Under anaerobic conditions, NreC de-represses genes involved in fermentative metabolism such as *lctE* (dissimilatory lactate dehydrogenase), *alsS* (2,3-butanediol pathway), and *nirBD*/respiratory *narGHJI* (nitrite and nitrate reductases) (Fedtke et al. 2002, Kamps et al. 2004, Schlag et al. 2007, 2008). These pathways are linked to the expression of polysaccharide intracellular adhesin production, thereby associating them with biofilm formation and persistent infections (Schlag et al. 2007, 2008).

Redox-responsive regulators: Rex monitors the redox state of the bacterium by responding to the relative concentrations of NADH and NAD^+, *i.e.*, the NADH/NAD^+ redox ratio (Brekasis and Paget 2003, Gyan et al. 2006, Sickmier et al. 2005). This sensor system functions both aerobically and anaerobically because the ratio of NADH/NAD^+ can change independently of oxygen, allowing it to monitor the redox state of the bacterium under a wide range of conditions (Brekasis and Paget 2003, Sickmier et al. 2005). When NAD^+ levels diminish, many bacterial dehydrogenases fail to function and the intracellular concentration of NADH increases relative to the NAD^+ concentration. Concomitant with an increased intracellular NADH concentration, Rex disassociates from its operator sites allowing gene activation. Rex regulates a number of genes directly or indirectly in response to oxidation of NADH, especially under anaerobic conditions (*nirR*, *nirC*, *ywcJ*, *srrA, adhE*, *adh1*, *alsS, lctE*, *lctP*, *pflB*, and *arcA*) (Hecker et al. 2009). Rex regulates *lukM*, a leukocidin gene (Hecker et al. 2009). Of particular interest is the de-repression of *srrAB*, which has been linked to toxin and biofilm production (see below).

Oxygen-dependent regulator: Staphylococcal response regulator (SrrBA) is a two-component histidine kinase-response regulator that influences the expression of RNAIII, TSST-1, protein A, and IcaR in response to oxygen availability (Pragman et al. 2004, 2007). In addition, it activates *icaABDC*, the operon involved in the biosynthesis of polysaccharide intercellular adhesin (Ulrich et al. 2007). This may occur due to shunting of carbohydrates toward glucosamine biosynthesis and away from the tricarboxylic acid cycle as SrrA represses aconitase, succinate dehydrogenase, and fumarase (Throup et al. 2001). UDP-*N*-acetylglucosamine is the biosynthetic precursor of PIA. We have recently demonstrated that PIA is synthesized when TCA cycle activity is repressed or blocked (Sadykov et al. 2008, Vuong et al. 2005); thus, the effect of *srrAB* on PIA biosynthesis is two-fold, direct regulation of *icaADBC* transcription and indirectly via repression of TCA cycle activity.

Oxidant-stress dependent regulator: MgrA, is a multiple gene regulator that positively affects the expression capsular polysaccharide and nuclease, represses the expression of α-toxin, coagulase, and protein A, and represses autolysis (Ingavale et al. 2003, Luong et al. 2003). MgrA contains a single cysteine (Cys12) in the dimerization domain (located in α-helix 1) that

is accessible to oxidizing agents. Because cysteinyl sulfhydryls can be oxidized to sulfenic acid by H_2O_2, it was postulated that MrgA could act as a sensor of peroxide stress (Chen et al. 2006). Oxidation of Cys12 to sulfenic acid results in the dissociation of MrgA with the *sarV* promoter, while the presence of reductants restores DNA binding activity (Chen et al. 2006).

3. Central Metabolism

The reciprocal regulation of secreted toxins and cell-associated adhesins correlates with TCA cycle activity. Specifically, when TCA cycle activity is repressed, adhesins are synthesized in abundance while the synthesis of secreted toxins is repressed. Several global regulators repress TCA cycle enzymes under conditions of nutrient abundance, (*e.g.*, CcpA, CodY, and SrrA) (Jourlin-Castelli et al. 2000, Kim et al. 2002, Seidl et al. 2008, Throup et al. 2001), but as nutrients become limiting, then non-preferred carbon sources (*e.g.*, acetate) are catabolized, RNAIII levels increase, and toxins are produced (Coulter et al. 1998, Mei et al. 1997, Sadykov et al. 2008, Smith et al. 1986, Somerville et al. 2002). The TCA cycle appears to affect virulence determinant biosynthesis on two levels: (*i*) Feedback inhibition of enzyme activity and (*ii*) Repression or activation of transcription. Interestingly, TCA cycle inactivation increased the transcription or stability of RNAIII. In addition to being a riboregulator, RNAIII encodes for δ-toxin, suggesting that TCA cycle inactivation would increase δ-toxin synthesis. This is not the case; TCA cycle inactivation strongly repressed δ-toxin accumulation in the culture supernatant. The reason that TCA cycle inactivation decreased δ-toxin synthesis was that the intracellular concentration of glutamate, derived from the TCA cycle intermediate α-ketoglutarate, prevented translation (Somerville et al. 2003). Supplementation of the culture medium with excess glutamate reversed this metabolic inhibition of δ-toxin translation, thus this example represents feedback inhibition of enzyme activity. In addition to creating a metabolic block, inhibiting TCA cycle activity dramatically increases *icaADBC* transcription and PIA biosynthesis (Sadykov et al. 2008, Vuong et al. 2005); thus, TCA cycle activity can alter transcription, perhaps by TCA intermediates acting upon regulatory proteins controlling PIA biosynthesis.

4. Conclusions

These examples of interactions between metabolism and virulence factor production show that the metabolic state of the bacterium has profound effects on multiple aspects of pathogenesis. This should not be surprising, as staphylococci must make many adjustments as the organism moves from the nasal mucosa to host tissues. In addition, the environment within the host changes, *e.g.*, the availability of nutrients and oxygen vary greatly from an initial infection to an abscess. These are profound environmental changes; hence, it is not surprising that the production of exoproteins and complex surface carbohydrates are influenced by these environmental changes.

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Festakt zur Ernennung der Deutschen Akademie der Naturforscher Leopoldina zur Nationalen Akademie der Wissenschaften

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Die Deutsche Akademie der Naturforscher Leopoldina wurde am 14. Juli 2008 im Rahmen eines Festaktes in Halle zur Nationalen Akademie der Wissenschaften ernannt. Damit erhielt Deutschland – wie andere europäische Länder oder die USA – eine Institution, die Politik und Gesellschaft wissenschaftsbasiert berät und die deutsche Wissenschaft in internationalen Gremien repräsentiert. Der Band dokumentiert den Festakt mit der Übergabe der Ernennungsurkunde durch die Vorsitzende der Gemeinsamen Wissenschaftskonferenz und Bundesministerin für Bildung und Forschung Annette SCHAVAN. Er enthält die Reden von Bundespräsident Horst KÖHLER, Sachsens-Anhalts Ministerpräsident Wolfgang BÖHMER und Leopoldina-Präsident Volker TER MEULEN sowie den Festvortrag „Rolle und Verantwortung nationaler Akademien der Wissenschaften“ von Jules A. HOFFMANN, Präsident der *Académie des sciences*, Paris. Der Aufbau einer Nationalen Akademie ist ein richtungsweisender Schritt für die deutsche Forschungslandschaft, da für den kontinuierlichen Dialog von Wissenschaft und Politik eine solche Einrichtung erforderlich wurde. Der Publikation ist eine DVD mit dem Mitschnitt der Festveranstaltung beigefügt.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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Sensing the World – How *Pseudomonas aeruginosa* Bacteria Assimilate and Process Environmental Signals

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With 1 Figure

Abstract

A number of bacteria that are common inhabitants of soil or water reservoirs can cause some of the most serious infections of humans. The ability of these opportunistic pathogens to thrive in a wide range of environments depends on the activities of specialized signal transduction pathways and complex regulatory networks that allow the bacteria to sense and respond to the presence of a human host. *Pseudomonas aeruginosa* can cause serious acute infections in immunocompromized individuals, or chronic infections in patients with cystic fibrosis. We have recently uncovered a regulatory network that, in response to yet unknown signals, functions as a molecular switch controlling the expression of hundreds of genes, including those encoding acute toxic proteins and, in a reciprocal mode, the formation of biofilm determinants important for chronic infections. The switch operates by controlling the reversible phosphorylation of the GacS/GacA two-component system and an orphan senor kinase RetS which interferes with GacS's phosphorylation activity by forming inactive heterodimers. The GacS/GacA/RetS system regulates transcription of only two genes, the small RNAs RsmZ and RsmY. The global impact of the GacS/GacA system is therefore due to post-transcriptional activities of these sRNAs, antagonizing the binding of the translational regulator RsmA to its target sites at the 5' ends of mRNAs. The mechanism of channeling signals through a limited number of two-component sensors and transmitting the information to the level of mRNA translation or stability appears to be a widely distributed mechanism of environmental response found in many opportunistic pathogens.

Zusammenfassung

Eine größere Anzahl von Bakterien, die normalerweise in der Umwelt (Erde, Wasser) leben, können auch schwere Infektionskrankheiten beim Menschen verursachen. Die Fähigkeit dieser opportunistischen Keime, sich an verschiedene Habitate inklusive den Menschen zu adaptieren, erfordert spezifische Signaltransduktionssysteme, um die jeweilige Umgebung des Errergers frühzeitig wahrzunehmen. *Pseudomonas aeruginosa* kann schwere akute Infektionen bei immun-inkompetenten Patienten und chronische Lungeninfektionen bei Mukoviszidosepatienten verursachen. Wir haben kürzlich ein Regulationsnetzwerk entdeckt, das über ein bisher noch unbekanntes Signalmolekül die Expression von Hunderten von Genen kontrolliert, darunter Gene für Toxine und Biofilmbildung. An dieser Regulation ist die reversible Phosphorylierung des Zweikomponentensystems GacS/GacA und der Sensorkinase RetS beteiligt, die mit der GacS-Kinaseaktivität interferiert, indem inaktive Heterodimere gebildet werden. Das GacS/GacA/RetS-System reguliert die Transkription von nur zwei Genen, den kleinen nichtkodierenden RNAs RsmZ und RsmY. Die globale Bedeutung des GacS/GacA-Systems liegt deshalb auf der posttranskriptionellen Aktivität der RNAs, die die Bindung des translationalen Regulators RsmA am 5'-Ende von mRNAs inhibieren. Die Signaltransduktion von Umweltsignalen über wenige Zweikomponentensysteme mit nachfolgender posttranskriptionaler Kontrolle der Pathogenität scheint ein typischer Mechanismus von opportunistischen Erregern zu sein.

1. Introduction

The adaptation of most microorganisms to life in a specific environment is accomplished by evolution of genomes containing a repertoire of genes encoding survival or fitness functions that allow them to utilize available nutrients and resist potential adverse conditions found in a particular niche. Moreover, survival also depends on their ability to coordinate the expression of groups of genes as dictated by the specific needs of the environment. Input signals provided by a particular environment are often assimilated through a diverse set of sensory proteins, often located in the bacterial cell envelope. External signals are transmitted to the level of gene expression by a variety of signal transduction pathways, typically modifying the activities of transcription factors. Adaptive responses of many prokaryotic organisms are mediated by two-component regulatory systems, consisting of sensor kinase and response-regulator pairs. Two-component systems are found not only in prokaryotic organisms and unicellular eukaryotes but also in higher plants (Wolanin et al. 2002). Two-component systems mediate downstream responses by a variety of mechanisms, including control of gene expression, enzymatic activities and methylation of target proteins (Galperin et al. 2001). The sensor histidine kinases detect a range of signals, including nutritional content of the environment, cation concentration and accumulation of denatured proteins during thermal stress. A number of two-component systems are used by pathogenic microorganisms to sense and respond to signals provided by infected hosts (Beier and Gross 2006).

The Gram-negative bacterium *Pseudomonas aeruginosa* is a common inhabitant of most tempered climate environments and is also an important human opportunistic pathogen capable of causing a wide range of infections. These include superficial skin infections, infections of wounds and burns, chronic respiratory disease in patients with cystic fibrosis (CF) and serious, often fatal, bacteremia in immunocompromised patients. This remarkable ability of an environmental organism to adapt to a variety of niches in humans and successfully overcome host defenses in different tissues depends to a large extent on its ability to coordinate the expression of genes necessary for survival in a particular environment and simultaneously repress genes that are not needed or whose expression could be detrimental. During human infections, the bacteria colonize tissues in a stepwise fashion, often encountering distinct niches that present specific challenges in terms of nutrient availability and the presence of tissue-specific host defense systems. Adaptation to each of the local environments requires the bacteria to coordinate the expression of blocks of genes through a network of regulatory factors acting at transcriptional and pos-transcriptional levels. Successful colonization of patients with CF requires the production of a variety of cytotoxic and degradative proteins, some of which, such as the proteins of the type III secretion system, function only after direct contact between *P. aeruginosa* and the host cells. These are the same virulence factors that play a key role in most acute infections. In CF, following the initial stage of colonization, bacteria switch to regulatory networks that facilitate the expression of cellular components that promote a biofilm lifestyle. Biofilms are matrix-enclosed communities where individual bacteria can no longer contact host cells and the virulence factors, such as secreted toxins, very likely play a smaller role. The expression of such toxic proteins is therefore reciprocally regulated with biofilm promoting factors.

One of the distinguishing features of the *P. aeruginosa* genome is the over-representation of transcriptional regulators in its genome (ca. 8 % of all genes), the highest percentage of

regulatory genes found in prokaryotic genomes (Stover et al. 2000). This group includes approximately 64 two-component systems, the most extensive repertoire of this family of signal transducing proteins known in a single organism (Rodrigue et al. 2000). This is not completely unexpected, as *P. aeruginosa* can thrive in a wide range of environmental niches and may therefore require a correspondingly large number of environmental sensing and signaling mechanisms. The presence of the large number of sensing and regulatory proteins raises the questions of whether *P. aeruginosa* responds to distinct environmental signals and whether the regulatory output is the consequence of the cumulative action of many two-component systems on groups of genes. Alternatively, the signal transduction pathways mediated by two-component systems may be more limited, acting through one or a few sensors and undergo amplification inside the cell, resulting in coordinate activation and repression of large blocks of genes. Here we describe our findings demonstrating the action of a two-component system, regulating the reciprocal expression of hundreds of acute and chronic virulence factors by a novel mechanism. Moreover, the regulatory output of this system is amplified at the post-transcriptional level, utilizing the activities of two small regulatory RNAs (sRNAs).

2. Two-Component Regulatory Switches Control the Reciprocal Expression of Virulence Factors Essential for Acute and Chronic Infections

Two independent approaches resulted in the identification of a network responsible for regulating the expression of various biofilm-promoting proteins and polysaccharides while reciprocally controlling the expression of secreted toxins. In a systemic study, generating mutations in most of the 64 response regulators or hybrid sensor kinases identified in the annotated genome of *P. aeruginosa*, we identified a gene, encoding a complex multimodular sensor kinase/response regulator pair, which reciprocally controls the expression of genes responsible for exopolysaccharide production and type III secretion (Goodman et al. 2004). A mutation in this gene (named *retS* for regulator of exopolysaccharide and type III secretion) resulted in a *P. aeruginosa* strain that showed an enhanced capacity to aggregate and formed robust biofilm in most *in vitro* assays. When examined for the ability to kill mammalian cells in culture, the *retS* mutant was completely non-cytotoxic due to the lack of expression of its type III secretion system. The *retS* mutant was also attenuated in a murine acute pneumonia model. In a separate study, a transposon mutagenesis screen was used to identify *P. aeruginosa* mutants defective in adherence to solid surfaces, and one such mutant was identified as another complex sensor kinase (Ventre et al. 2006). This mutant was named *ladS* (for lost adherence sensor) and many of its phenotypes mirror those of the *retS* mutation. In addition to a defective adhesion and biofilm formation, the expression of genes for type III secretion was significantly increased when compared to the wild type. A comparison of *retS* and *ladS* transcriptomes confirmed that the regulatory control of the products of these two gene products is indeed reciprocal, with the levels transcripts of several hundred shared genes altered.

One of the striking features of the RetS and LadS polypeptides is their similarity in domain organization. They both appear to be integral cytoplasmic membrane proteins with a periplasmic domain flanked by an N-terminal transmembrane segment and seven transmembrane segments, referred to in the PFAM database as a 7TMR-DISMED2 domain. In both

RetS and LadS, this domain is linked to the histidine kinase transmitter domain followed by a receiver domain. However, RetS also contains an additional response regulator domain. Neither the *ladS* and *retS* genes are linked to cognate response regulators, and this unusual arrangement suggests that they exert their regulatory activities by interacting with another two-component system. This hypothesis has been validated for RetS (see below).

The final element of the regulatory system controlling the reciprocal regulation of chronic and acute virulence factors is the two-component system GacS (sensor) and GacA (response regulator), which was identified in a screen for suppressors of phenotypes of the *retS* mutants (Goodman et al. 2004). The orthologues of GacA are distributed in many disease-causing bacteria including plant pathogens (Haas and Keel 2003), where they appear to control a diverse set of virulence genes. The GacS/GacA system also controls the expression of small regulatory RNAs of the Csr/Rsm family (Valverde and Haas 2008) and one of the mutations that suppressed the *retS* phenotypes in various screens was in the *P. aeruginosa rsmZ* gene (Goodman et al. 2004).

3. Mechanism of Regulation of Virulence Factor Expression by the RetS/Lads/GacSA Signal Transduction System

Microarray analyses and the phenotypes of various mutants suggested that the regulatory pathways controlled by two orphan sensor kinases RetS and LadS flow through the GacS/GacA two-component system, where RetS antagonizes and LadS enhances the regulatory output of the GacS/GacA system. We were able to demonstrate that GacS forms dimers as the prerequisite for autophosphorylation at specific histidine residues of each monomeric subunit from which the phosphate is transferred to GacA. RetS interferes with the formation of active GacS homodimers (Goodman et al. 2009). Several *in vitro* and *in vivo* protein-protein interaction assays were used to demonstrate that RetS and GacS directly interact and form a heterodimer, suggesting that the sensor kinase RetS exerts its regulatory activity through a direct and specific interaction with GacS, interfering with its auto-phosphorylation and, presumably, limiting phosphorylation of GacA, the transcriptional activator of *rsmZ* and *rsmY* sRNAs genes. This specific interaction does not require the presence of any of the canonical catalytic or phosphoacceptor residues in the histidine kinase and response regulator domains of RetS. The model for regulation of gene expression by the interplay between RetS and the GacS/GacA two-component system is shown schematically in Figure 1.

The model where a sensor kinase interferes with the kinase activity of another sensor kinase provides a novel mechanism for controlling a signal transduction network mediated by two-component systems. Both GacS and RetS contain domains associated with signal reception, the HAMP domain and the 7TMR-DISMED2, respectively. It is conceivable that the GacS autokinase activity responds to one set of signals while heterodimerization between GacS and RetS is induced by another signal received by the periplasmic 7TMR-DISMED2 domain of RetS. To date, none of the characterized two-component systems have been shown to be regulated by the mechanism described for the *P. aeruginosa* GacS and RetS.

Two regulatory targets of GacS, Rets and LadS are the genes of regulatory small RNAs RsmY and RsmZ (Ventre et al. 2006, Godman et al. 2009). These sRNAs control gene expression at the post-transcriptional level, by modulating the activity of the translational repressor RsmA. The main function of the RsmA protein is to bind to 5' untranslated regions

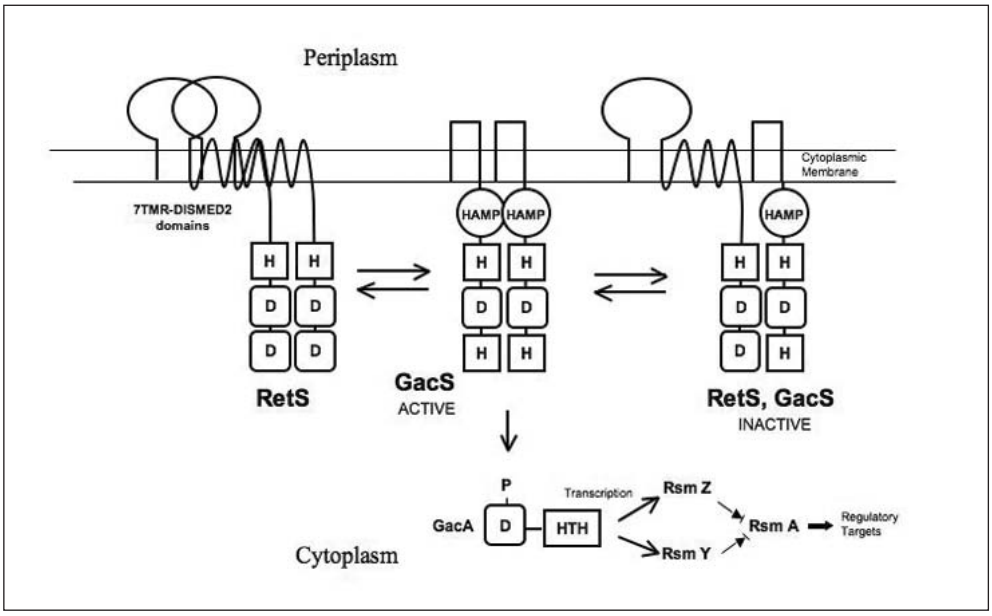

Fig. 1 A model for regulation of expression of small regulatory RNAs by GacS/ GacA two-component system. Phosphorylation of GacA, the transcription factor required for the expression of RsmZ and RsmY, is blocked by RetS sensor kinase by forming an inactive heterodimer with GacS. The sRNAs interfere antagonize the post-transcriptional regulatory ativity of RsmA. The histidine kinase and response regulator domains are designated as H and D, respectively.

of target mRNAs and interfere with translational initiation, facilitating mRNA degradation. In a few instances, RsmA stabilizes transcripts. The RsmY, RsmZ sRNAs function by sequestering RsmA from its mRNA targets, thus reversing the effect of this protein on translation and mRNA stability. Since the *rsmY* and *rsmZ* genes are regulated by the GacS/GacA system, the deletion of the regulatory sRNAs genes in *P. aeruginosa* leads to phenotypes that are similar to those of mutants in this two-component system and mirror the phenotypes seen in the *retS* mutant. By comparing transcriptomes of various mutants in the signal transduction pathways, we have confirmed that the regulatory consequences on mRNA levels in *gacA*, *retS*, and *rsmZY* mutants are nearly completely overlapping, suggesting that the GacS/GacA signal transduction pathway acts primarily through its control over the expression of the sRNAs (Brencic and Lory 2009).

To examine the range of genes regulated by phosphorylated GacA we carried out a genome-wide DNA-protein interaction analysis and identified only two genomic regions located upstream of the *rsmY* and *rsmZ* genes that associated specifically with GacA. These results demonstrate that in *P. aeruginosa*, the regulatory signals transduced by the GacS/GacA pathway or system are channeled exclusively into the transcription of only two promoters controlling expression of RsmY *and* RsmZ. These two regulatory RNAs serve as intermediates between the signal received by GacS and the output at the level of mRNA stability, although additional regulatory inputs can influence their levels and activities. We have shown that the DNA segment upstream of the *rsmZ* gene is highly A+T-rich and it is bound and silenced by MvaT and MvaU, the global gene regulators of the H-NS family. Therefore, it is apparent that

post-transcriptional mechanisms involving sRNAs in controlling gene expression play a key role during bacterial adaptation to different environments.

4. Summary and Perspective

The work presented here suggests a simple model (shown schematically in Figure 1) for global regulation of gene expression in response to environmental stimuli during various phases of infection by *P. aeruginosa*. The signaling network feeds into a post-transcriptional mechanism, controlled by the action of two regulatory sRNAs, RsmY and RsmZ, that influence the translation and/or stability of nearly 500 transcripts. The evolutionary rationale favoring post-transcriptional regulation mediated by only two molecules (sRNAs), as opposed to direct regulation of gene expression by more traditional mechanisms through the activity of transcription factors, is unclear. The identities of the environmental signals controlling the activities of the GacS, RetS and LadS two-component sensors remain the critical missing pieces of information that need to be discovered before we can fully understand the molecular details of the workings of the signaling pathway that appears to be widely distributed among opportunistic pathogens capable of thriving not only in environmental reservoirs, but also in human, animal or plant hosts.

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Gedenken an die Leopoldina-Mitglieder, die in Konzentrationslagern des nationalsozialistischen Regimes zu Tode kamen

Einweihung einer Gedenkstele am 1. Oktober 2009 in Halle (Saale)

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Zum Andenken der Mitglieder der Deutschen Akademie der Naturforscher Leopoldina, die in nationalsozialistischen Konzentrationslagern oder an den Folgen der Lagerhaft zwischen 1942 und 1945 ums Leben kamen, wurde in Halle im Oktober 2009 eine Gedenkstele enthüllt. Der vorliegende Band dokumentiert die Veranstaltung und setzt damit auch das Bestreben der Akademie fort, in Publikationen und Vorträgen die Geschichte der Leopoldina in den Jahren zwischen 1933 und 1945 aufzuarbeiten. Dem kulturellen Gedächtnis der Leopoldina wird mit der Gedenkstele ein weiterer Mosaikstein hinzugesetzt, weil die Entstehung, die Entwicklung und die Bewahrung einer Erinnerungskultur zu den Grundlagen unserer Freiheit und damit zu den Grundlagen der Demokratie in Deutschland gehören. Die Beiträge sind in deutscher und englischer Sprache verfasst.

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Escherichia coli Metabolism in the Intestine

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With 2 Figures and 1 Table

Abstract

Pathogenic *Escherichia coli* may compete for nutrient-defined niches that can be occupied by commensal *E. coli* strains. We have found that several human commensal *E. coli* strains grow from low to high numbers and co-colonize mice that have been pre-colonized with another *E. coli* strain. Our results suggest that *E. coli* expands or modifies the niches it occupies by diversifying its nutrition in response to competition. Moreover, although *E. coli* O157:H7 is able to either persist in low numbers or grow from low to high numbers, depending on which pre-colonized human commensal *E. coli* strain is present, a combination of human commensal *E. coli* strains can completely prevent *E. coli* O157:H7 colonization. Thus, we hypothesize that the more fully filled the intestinal niches occupied by commensal *E. coli* are, the more effective the barrier to infection against *E. coli* enteric pathogens.

Zusammenfassung

Pathogene *Escherichia coli* konkurrieren um Nährstoffe mit kommensalen *E. coli* in gemeinsamen Habitaten wie z. B. dem Darm. Wir haben herausgefunden, dass sich einige kommensale *E. coli*-Stämme besser im Darm vermehren, wenn dieser bereits mit anderen *E. coli*-Stämmen kolonisiert war. Unsere Ergebnisse lassen vermuten, dass das Nahrungsangebot für konkurrierende *E. coli*-Stämme durch Ko-Kolonisierung verbessert wird. Die Keimkonzentration von enterohämorrhagischen *E. coli* O157:H7 (EHEC) im Darmlumen kann durch Kombinationen bestimmter kommensaler *E. coli*-Stämme moduliert werden, bis hin zur vollständigen Inhibition der Darmkolonisierung mit EHEC. Daraus schließen wir: je vollständiger die Kolonisierung von Darmhabitaten mit kommensalen *E. coli*, desto größer ist der Schutz vor Infektion mit EHEC.

1. Introduction

E. coli represents 0.02 % of intestinal bacteria. Human feces contain a microbial community comprised of thousands of different species and a total of 5×10^{11} bacteria per g. We calculate a global population of 10^{22} *E. coli* cells in human gastrointestinal tracts. *E. coli* is the best understood of any organism on the planet, but as Falkow states, "No one has yet worked out the essence of the biology of *why* or *how E. coli* colonizes the bowel as a commensal" (Relman and Falkow 2001). Our research is beginning to reveal the answers, many of which are summarized in several review articles (Conway et al. 2004, Laux et al. 2005, Conway et al. 2007). "Why" *E. coli* colonizes has to do with the symbiotic *E. coli*–host relationship: the host creates an environment favorable for the growth of *E. coli,* and *E. coli* creates an anaerobic environment favorable for the anaerobes that are so important for intestinal health (Jones et al. 2007). "How" *E. coli* colonizes has to do with its ability to grow on the mixture

of simple sugars that are made available by both the host and the activities of the anaerobic microbiota (Fabich et al. 2008).

1.1 Freter's Nutrient-Niche Hypothesis

Much of our work is predicated on Freter's nutrient-niche hypothesis (Freter 1983, 1988, 1992). In short, the numerous ecological niches within the intestine are defined by nutrient availability. For different species to coexist in the intestine each must be able to utilize a limiting nutrient better than any other species; their growth rates must equal or exceed the washout rate; species that do not compete well for limited nutrients can colonize if they are able to adhere to the intestinal wall; colonization resistance to a specific strain occurs when all of its niches are occupied. Of the several barriers to colonization, Freter concluded that competition for nutrients is the most important. We have made extensive use of the streptomycin-treated mouse model to measure the relative fitness of wild-type *E. coli* and isogenic catabolism mutants to determine which nutrients support *E. coli* colonization of the intestine (Conway et al. 2004, Laux et al. 2005, Conway et al. 2007, Leatham et al. 2009).

1.2 The Streptomycin-treated Mouse Model

Streptomycin-treated mice have been used since 1954 to overcome the colonization resistance encountered in conventional animals (Bohnhoff et al. 1954). Mice are given streptomycin sulfate in their drinking water (5 g/L) during the entire course of the experiment which selectively removes facultative anaerobic *E. coli*, enterococci, streptococci, lactobacilli, and anaerobic lactobacilli and bifidobacteria (Hentges et al. 1984). Nevertheless, the overall population of anaerobes, including *Bacteroides* and *Eubacterium*, in the cecal contents following streptomycin treatment is unchanged. Therefore, the streptomycin-treated mouse model allows for colonization by experimentally introduced *E. coli* strains (streptomycin resistant) and competition with large numbers of strict anaerobes, and thus is our model of choice for studying competition among *E. coli* strains in the intestine, the details of which are provided elsewhere (Conway et al. 2004). Since the numbers of a strain of *E. coli* in mouse feces is a reflection of its numbers in the mouse large intestine (Lee 1985), fecal counts are used to judge the relative colonizing abilities of various *E. coli* strains.

2. Colonization Experiments

2.1 E. coli Growth on Sugars

The results of many competitive colonization experiments (Sweeney et al. 1996, Chang et al. 2004, Miranda et al. 2004, Autieri et al. 2007, Fabich et al. 2008, Jones et al. 2008) for two *E. coli* commensal strains and one pathogen are summarized in Table 1.

Different *E. coli* strains generally have the same metabolic capacity for growth on sugars *in vitro*, but appear to use different sugars in the mouse intestine, i.e., not all strains occupy all available niches (Fabich et al. 2008). We found that *E. coli* EDL933 and *E. coli* MG1655 both use arabinose, fucose, maltose, and *N*-acetylglucosamine in the mouse intestine, but *E. coli* EDL933 uses 3 sugars not used by *E. coli* MG1655 (galactose, mannose, and ribose), whereas *E. coli* MG1655 uses 2 sugars not used by *E. coli* EDL933 (sialic acid and gluco-

Tab. 1 Colonization defects of catabolism mutants in competition with WT parent

Sugar-negative phenotype	Mutation	MG1655	Nissle 1917	EDL933
Arabinose	*araBAD*	Yes	Yes	Yes
Fucose	*fucAO*	Yes	Yes	Yes
Galactose	*qalK*	No	Yes	Yes
Gluconate	*gntK* (*idnK*)	Yes	Yes	No
Hexuronates	*uxaC*	No	Yes	Yes
Lactose	*lacZ*	No	No	No
Maltose	*malQ*	Yes	Yes	Yes
Mannose	*manA*	No	Yes	Yes
N-Acetylglucosamine	*nagE*	Yes	No	Yes
N-Acetylgalactosamine	*agaWEFA*	No Pathway	Yes	No
Sialic acid	*nanAT*	Yes	No	No
Ribose	*rbsK*	No	No	Yes
Sucrose	*sacH*	No Pathway	No Pathway	Yes

Yes: > 1 log, $P < 0.05$

nate). Moreover, different *E. coli* commensals use different nutrients in the mouse intestine: i.e., *E. coli* MG1655 and *E. coli* Nissle 1917 both use arabinose, fucose, gluconate and maltose; *E. coli* Nissle 1917 also uses D-mannose and galactose, which are not used by *E. coli* MG1655; *E. coli* MG1655 uses *N*-acetylglucosamine and sialic acid, which are not used by *E. coli* Nissle 1917. Thus, different *E. coli* commensal strains may compete for different niches in the intestine. Importantly, it appears that pathogenic *E. coli* might compete for niches that are available, or not, depending on the metabolism of the resident commensal *E. coli* strains.

2.2 Metabolic Strategies Increase Colonization Fitness

The intestine selects for strains that are better colonizers. Following colonization, within seven days 80–90 % of *E. coli* MG1655 become non-motile (Leatham et al. 2005). Each non-motile isolate from the intestine was found to have an *flhDC* deletion, beginning with the upstream IS1 element. Further investigation revealed that strains lacking the flagellar master regulator, FlhDC, have a colonization advantage over the wild-type for two reasons: (*i*) the *flhD* mutation de-represses many catabolic pathway genes that maximize colonization and (*ii*) the energy that otherwise would be used for flagellar synthesis and rotation is redirected to growth (Gauger et al. 2007). From these results we conclude that metabolic strategies that improve energy efficiency increase colonization fitness.

2.3 E. coli Strains Adapt to One Another by Expanding or Modifying their Niches

We found that fucose negative mutants switch to ribose in the intestine (Autieri et al. 2007). Furthermore, fucose stimulates ribose catabolism *in vitro*, and fuculose-1-phosphate is required for the stimulation. From these results we conclude that *E. coli* strains may adapt to one another by expanding or modifying the niches they occupy, resulting in a more stable commensal *E. coli* microbiota.

2.4 Nutritional Diversity

FRETER's hypothesis states that each intestinal species uses one, or a few, nutrients better than all others nutrients and, while this is essentially correct, it is apparently oversimplified. Different *E. coli* strains are distinct nutritional biotypes, each using multiple substrates. *E. coli* has the potential to adapt to the intestine to become a better colonizer, e.g., *flhDC* mutants. *E. coli* can switch to alternative nutrients when preferred nutrients are missing, e.g., fucose-ribose switch. Together, these results indicate a previously unsuspected nutritional diversity that apparently allows several *E. coli* strains to co-colonize the intestine and thereby occupy all available niches.

2.5 Glycogen and Colonization

Endogenous glycogen appears to play an important role in colonization, since mutants that are unable to synthesize or degrade glycogen have significant colonization defects (JONES et al. 2008). In support of the hypothesis that *E. coli* relies on internal carbon stores to maintain colonization during periods of famine, we found that by providing a constant supply of a readily metabolized sugar, i.e., gluconate, in the animal's drinking water, the competitive disadvantage of *E. coli* glycogen metabolism mutants is rescued. The results suggest that glycogen storage may be widespread in enteric bacteria because it is necessary for maintaining rapid growth in the intestine, where there is intense competition for resources and occasional famine. An important implication of this study is that the sugars used by *E. coli* are present in limited quantities in the intestine, making endogenous carbon stores valuable.

2.6 The Role of Respiration in Colonization

Respiration supports *E. coli* colonization of the intestine (JONES et al. 2007). A mutant lacking ATPase is completely eliminated in competition with the wild-type, but is able to colonize when it alone is fed to mice. This means that respiration is essential for competitive fitness in the intestine, but fermentation is sufficient for colonization. We found that mutants lacking cytochrome *bd* oxidase, nitrate reductase, or fumarate reductase were completely eliminated in competition with the wild-type, suggesting that the intestinal environment is alternately microaerobic and anaerobic. As shown in Figure 1, a mutant lacking fumarate reductase initially outcompeted a mutant lacking nitrate reductase, but subsequently was eliminated from the intestine. In this experiment, the population of the strain lacking fumarate reductase (possessing nitrate reductase) was correlated with nitrate availability. This experiment reveals that nitrate is limiting in the intestine. Together, these results support the conclusion that *E. coli* maximizes its cell yield (population) in the intestine by respiring carbon sources that are apparently available only in limited amounts.

3. Results

3.1 The Model of Colonization Resistance

Colonization resistance can be modeled in the streptomycin-treated mouse intestine (LEATHAM et al. 2009). When mice were pre-colonized with any one of three human commensal strains,

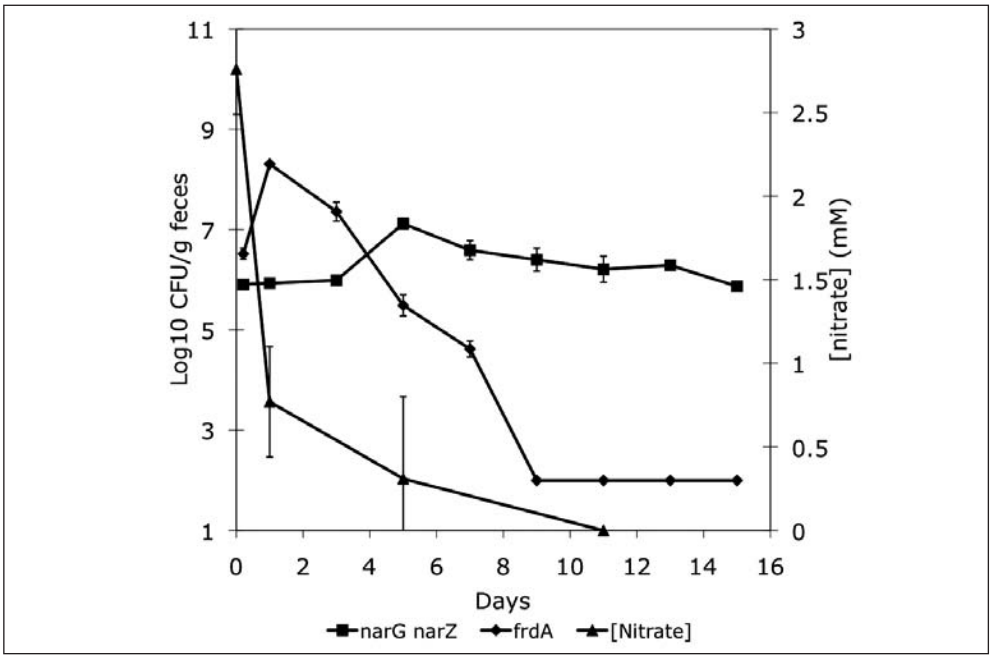

Fig. 1 A mutant lacking fumarate reductase initially outcompetes a mutant lacking nitrate reductase, but subsequently is eliminated from the intestine. Competitiveness of the strain lacking fumarate reductase but possessing nitrate reductase correlated with nitrate availability. Six mice were fed 10^5 CFU each of *E. coli* MG1655 Δ*narG* Δ*narZ* and *E. coli* MG1655 Δ*frdA* and CFU/g feces determined at the indicated times. Nitrate in cecal mucus was determined for three mice at the indicated times under identical experimental conditions.

E. coli MG1655, *E. coli* HS, or *E. coli* Nissle 1917, and 10 days later were fed 10^5 CFU of any one of the strains, the pre-colonized strain nearly eliminated its isogenic strain. On the other hand, each commensal strain allowed growth of the other commensal strains to higher numbers. This result is consistent with different commensal *E. coli* strains using different nutrients in the intestine.

3.2 Limitation of Colonization

E. coli commensal strains differ in their ability to limit colonization by *E. coli* EDL933, an O157:H7 pathogen (Leatham et al. 2009). We found that *E. coli* Nissle 1917 and *E. coli* EFC1 limited growth of *E. coli* EDL933 in the intestine (10^3–10^4 CFU/g of feces), whereas *E. coli* MG1655, *E. coli* HS, and *E. coli* EFC2 allowed growth to higher numbers (10^6–10^7 CFU/g of feces). Importantly, when *E. coli* EDL933 was fed to mice previously co-colonized with three *E. coli* strains, MG1655, HS, and Nissle 1917, it was eliminated from the intestine (<10 CFU/g of feces) (Leatham et al. 2009). As shown in Figure 2, *E. coli* EDL933 was also completely eliminated from mice that were pre-colonized with *E. coli* strains Nissle 1917, EFC1, and MG1655. These results confirm that commensal *E. coli* strains can provide a barrier to infection and suggest that it may be possible to construct *E. coli* probiotic strains that prevent growth of pathogenic *E. coli* strains in the intestine.

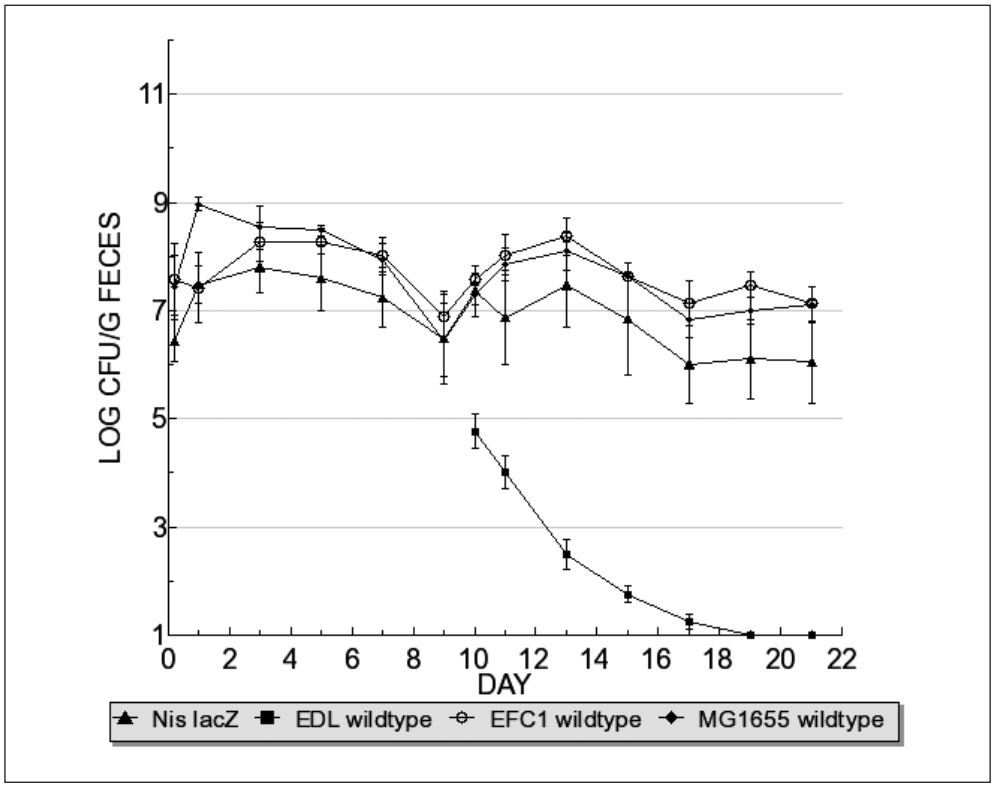

Fig. 2 *E. coli* EDL933 is completely eliminated from the intestine that is pre-colonized with several commensal *E. coli* strains. Mice were also pre-colonized with 10^5 CFU each of three commensal strains, *E. coli* Nissle 1917, *E. coli* EFC1, and *E. coli* MG1655, for 10 days and then were fed 10^5 CFU of *E. coli* EDL933.

4. Conclusion

In summary, we conclude that the physiology of *E. coli* is tuned to life in the intestine. *E. coli* is well suited to the nutrient-limiting conditions of the intestine and therefore is a remarkably successful colonizer in a symbiotic relationship with the host. *E. coli* is one species, consisting of many nutritional biotypes. Apparently, each biotype uses one or more sugars better than others sugars. Collectively, the commensal *E. coli* microbiota may form a barrier to enteric disease by limiting nutrient availability to invading pathogens. Thus, it might be possible to identify the characteristics (e.g., carbon catabolism) that make each *E. coli* strain a successful colonizer and use genetics to combine them in a single strain that serves as a "probiotic" to prevent colonization by pathogens and thus short circuit the infection process.

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Carbon Metabolism of Intracellular Bacterial Pathogens within Mammalian Cells

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Abstract

Studies on the intracellular carbon metabolism of *Listeria monocytogenes*, enteroinvasive *Escherichia coli* and *Salmonella typhimurium* after infection of J774 and Caco-2 cells are presented and possible interactions of the central virulence gene regulator of *L. monocytogenes* with PTS permeases are discussed. C metabolism of the bacterial pathogens was analyzed by ^{13}C isotopologue profiling.

Zusammenfassung

Mit Hilfe von NMR- und MS-basierten ^{13}C-Isotopolog-Analysen wurde der intrazelluläre Kohlenstoffmetabolismus von *Listeria monocytogenes*, enteroinvasiven *Escherichia coli* und *Salomonella typhimurium* nach Infektion von J774 und Caco-2-Zellen untersucht. Die Ergebnisse dieser Analysen werden ebenso diskutiert wie die möglichen Interaktionen des zentralen Virulenzregulators von *L. monocytogenes*, PrfA, mit Komponenten von bestimmten listeriellen PTS-Permeasen.

1. Introduction

Infections of mammals by intracellular bacterial pathogens require multiple metabolic adaptations by the bacteria. However, our knowledge of this aspect of bacterial pathogenesis is still rather limited (Munoz-Elias and McKinney 2005, Ray et al. 2009).

The *in vivo* metabolism of bacterial pathogens is, in general, difficult to study with the classic methods, but several novel techniques, such as signature-tagged mutagenesis (Saenz and Dehio 2005), high-throughput methods, particularly the differential gene expression profiling (DGEP) (Jansen and Yu 2006), metabolite profiling (Nielsen and Oliver 2005), the ^{13}C isotopologue profiling analysis (^{13}C-IPA) (Sauer 2006, Eylert et al. 2008), Raman microspectroscopy (Swain and Stevens 2006), and various imaging techniques (Melican and Richter-Dahlfors 2009) now allow first insights into the bacterial metabolism within host cell and animal models. In our contribution we focus on the carbon metabolism of three intracellular bacterial pathogens, namely *Listeria monocytogenes*, enteroinvasive *Escherichia*

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coli (*EIEC*) and *Salmonella enterica* serovar Typhimurium which replicate in the host cell's cytosol and the *Salmonella*-containing vacuole (SCV), respectively. We will also address some interesting links between the intracellular C metabolism and the activity of the central virulence regulator, PrfA, which controls the expression of the major virulence genes of *L. monocytogenes.*

2. Results and Discussion

2.1 The Metabolic Potentials of the Three Intracellular Bacterial Pathogens as Deduced from their Genome Sequences and from In vitro Growth Studies

The genomes of *Shigella flexneri* (Jin et al. 2002), highly related to that of *EIEC*, and of *S. typhimurium* (Bell et al. 2004) show complete gene sets for all major catabolic and anabolic pathways – typical of a prototrophic/heterotrophic microorganism. In contrast, the *Listeria monocytogenes* genome lacks essential genes of the citrate cycle, the genes of the glyoxylate shunt and other anaplerotic reactions, as well as the genes for the biosyntheses of biotin, thiamine, riboflavin and lipoate. Most *fad* genes, essential for the degradation of fatty acids, are also absent (Glaser et al. 2002).

Therefore, growth of *L. monocytogenes* in defined minimal media – in contrast to *EIEC* – requires, in addition to a suitable carbon source, not only the four vitamins (as anticipated from the genomic information), but also Met, Cys and the three branched chain amino acids Val, Ile and Leu (Premaratne et al. 1991). The growth dependency on the latter amino acids is probably a consequence of the disrupted citrate cycle (Eisenreich et al. 2006). Due to the lack of the genes for the oxoglutarate dehydrogenase (SUC) and malate dehydrogenase (MDH), *L. monocytogenes* generates oxaloacetate predominantly by pyruvate carboxylation catalyzed by ATP-dependent pyruvate carboxylase (PYC). This anaplerotic reaction is crucial in the C metabolism of *L. monocytogenes* as demonstrated by the inability of a *pycA* insertion mutant to grow in defined minimal growth media regardless of the carbon source used and within mammalian host cells (Schär et al. 2010).

All three pathogens use, for *in vitro* growth, glucose as a preferred carbon source which is taken up by the two enterobacteria mainly via two PTS permeases (PtsG/Crr and ManXYZ) (Götz et al. 2010). *L. monocytogenes* lacks a PtsG homologous permease (the major glucose-specific PTS transporter in most heterotrophic bacteria) and glucose is taken up by several PTS permeases (PTS^{Man} and PTS^{Glc}) which are expressed under different conditions (Stoll und Goebel 2010). The affinity of these PTS permeases for glucose is, however, at least 10 times lower than that of the PtsG permeases from *EIEC* or *Salmonella* (A. Götz, personal communication), i.e. growth of *L. monocytogenes* at low glucose concentration will be less efficient than that of the two enterobacteria.

Glucose-6 phosphate can be also directly taken up from the environment by all three pathogens via the UhpT transporter. The UhpT transporter protein is highly homologous in these bacteria, but the regulation of the *uhpT* gene is remarkably different: in *L. monocytogenes* the *uhpT* gene is under the control of PrfA (Chico-Calero et al. 2002), the central transcriptional regulator of the major listerial virulence genes (see below), whereas in *EIEC* and *Salmonella* the *uhpT* gene is under the control of a complex two-component system (*uhpABC*) (Verhamme et al. 2002). The listerial as well as the enterobacterial *uhpT* genes are

additionally controlled by carbon catabolite repression (CCR) and hence hardly expressed in the presence of glucose.

Glycerol is also a suitable C_3 carbon source for these pathogens, and the genes for the facilitated uptake (*glpF*) and the catabolism (*glpK* and *glpD*) of glycerol are highly homologous, and the expression of these genes is also under CCR control (Joseph et al. 2008). Noteworthy, *L. monocytogenes* has two differently regulated genes for glycerol uptake facilitators and glycerol kinases which seem to contribute differently to intracellular growth (Joseph et al. 2008). However *L. monocytogenes* lacks, in contrast to *EIEC* and *S. typhimurium*, a *glpT* gene for glycerol-3P uptake.

L. monocytogenes possesses also genes for two dihydroxyacetone kinases (Barabote and Saier 2006, Joseph et al. 2008), a glycerol dehydrogenase and a glycerol dehydratase (propandiol dehydratase) which may widen the range of the C_3 metabolism. With the exception of a single gene for dihydroxyacetone kinase, these genes are absent in the two enterobacteria.

L. monocytogenes, other than *EIEC* and *S. typhimurium*, is unable to grow solely on C_2- (and C_4-) substrates due to the missing glyoxylate shunt and the disrupted citrate cycle, respectively (Schär et al. 2010). Hence *L. monocytogenes* cannot utilize fatty acids as a carbon source.

2.2 Intracellular Bacterial Metabolism of the Three Bacterial Pathogens as Deduced from ^{13}C Isotopologue Profiling

The intracellular metabolism of *L. monocytogenes*, of two EIEC strains and a *S. typhimurium* strain, replicating either in J774 macrophages or Caco-2 epithelial cell, was studied by ^{13}C isotopologue profiling using [U-$^{13}C_6$]glucose (Sauer 2006, Eylert et al. 2008). In short, the bacteria were grown in these mammalian host cells in the presence of uniformly labelled ^{13}C glucose. Then, all amino acids from the bacteria and the host cells were isolated and those amino acids that were labelled with ^{13}C (and hence *de novo* synthesized by the bacteria or the host cells) were further analyzed for their ^{13}C distribution patterns (i.e. whether they contained $^{13}C_1$-, $^{13}C_2$-, $^{13}C_3$-, $^{13}C_4$-, etc. isotopologues) by NMR or mass spectrometry (MS). Since all amino acids derive from intermediates of the central catabolic pathways (mainly glycolysis, pentose phosphate cycle, citrate cycle), the ^{13}C profiles of the *de novo* synthesized bacterial and host cell amino acids allow the deduction of the carbon substrates utilized for the intracellular bacterial C metabolism, the reconstruction of the catabolic pathways employed by the intracellular bacteria and the host cells as well as the determination of the amount of host cell-derived amino acids imported by the intracellularly growing bacteria.

In the following the major results of these studies are summarized: *L. monocytogenes* multiplied in J774 cells by using mainly glycerol and to a lesser extent glucose-6P as a carbon source while glucose was not utilized (Eylert et al. 2008). A major role in the intracellular listerial metabolism played the pyruvate carboxylase (PYC) which, due to the incomplete citrate cycle of *L. monocytogenes*, is essential for the conversion of C_3- to C_4- and C_5- metabolites.

The determination of the intracellular carbon metabolism of two *EIEC* strains, replicating in Caco-2 cells, showed in one strain the preferential utilization of glucose as substrate for intracellular growth, while glucose-6P was not used. Mutants impaired in the uptake of glucose switched to C_3 carbon source(s) and imported increased amounts of amino acids

from the host cell. The second *EIEC* strain utilized glucose less efficiently, produced fewer amino acids and imported larger amounts of amino acids from the host cell. The ^{13}C profiles of the amino acids synthesized by this wild-type strain and by a mutant defective in glucose/glucose-6P transport were rather similar and suggested the preferential use of C_3 substrate(s) for intracellular growth.

S. typhimurium, although replicating in a phagosomal compartment, showed a similar C metabolism as the first EIEC strain by using primarily glucose as a carbon source and C_3 substrate(s) as an alternative carbon source when the uptake of glucose was impaired.

Thus, all three intracellular bacterial pathogens showed a high flexibility in the utilization of carbon substrates for their intracellular C metabolism and the uptake of significant, but highly varying, fractions of host cell-derived amino acids for their protein synthesis. The replacement of a high-energy by a lower-energy carbon substrate seems to be balanced by an increased uptake of host cell amino acids and possibly other anabolic monomers (Götz et al. 2010).

2.3 The PTS Permeases of L. monoctogenes and their Interaction with the Central Virulence Regulator PrfA

Previous studies suggested an interaction of the PEP-dependent phosphotransferase systems (PTS), mainly responsible for the uptake of carbohydrates in *L. monocytogenes* with the central transcriptional activator of most virulence genes (Mertins et al. 2007, Stoll et al. 2008). To study these interactions on a biochemical basis, we started to analyze the listerial PTS in more detail (Stoll und Goebel 2010). To summarize the present state of these studies: *Listeria monocytogenes* EGD-e possesses 86 *pts* genes encoding 29 complete and several incomplete PEP-dependent phosphotransferase systems (PTS) for the transport of carbohydrates and sugar alcohols. By a systematic deletion analysis we identified the major PTS involved in glucose, mannose and cellobiose transport when *L. monocytogenes* grows in a defined minimal medium in the presence of either of these carbohydrates. Under these conditions, two of the four PTS^{Man} and at least one of the five PTS^{Glc} function as a glucose transporter with different affinities. Cellobiose transport is carried out (with different efficiencies) mainly by two of the six PTS^{Lac} and by the same PTS^{Glc} that can also take up glucose. One of the PTS^{Man} and both PTS^{Lac} are regulated by LevR-homologous PRD containing transcriptional activators. The growth rate of mutants that have lost these PTS is drastically reduced when the bacteria grow in a defined minimal medium with glucose or cellobiose. In contrast, replication of these PTS mutants within epithelial cells or macrophages is as efficient as that of the wild-type strain.

Preliminary data indicate that the EIIA components of the main PTS permeases of *L. monocytogenes* interact strongly with PrfA, blocking the activity of PrfA, when these EIIA components are unphosphorylated (i.e. during active uptake of carbohydrates) and activating PrfA, when the EIIA components are in the phosphorylated state.

3. Conclusions

The intracellular C metabolism of the three bacterial pathogens studied show strain-specific metabolic adaptations when these bacteria grow in actively proliferating J774 or Caco-2 cells,

suggesting that the intracellular milieu encountered by the pathogens in the host cells induces strain-specific metabolic adaptations.

The C metabolism of the intracellular pathogens shows high flexibility: when a preferentially utilized carbon source becomes unavailable, the bacteria can readily switch to second-choice carbon substrates.

At least under the rich culture conditions applied in the reported studies, the switch in C substrates causes relatively little change in the growth rate of the respective pathogen. This seems to be achieved by adapting the bacterial anabolic metabolism to the proffered carbon source, i.e. the exchange of an energy-rich C substrate by a lower energy C substrate(s) is balanced by an increased import of amino acids from the host cells.

Glucose and C_3 substrate(s), as well as amino acids, are not only readily accessible to cytosolically replicating intracellular bacteria but can be also transported into the SCV.

Further studies with more refined host systems, such as appropriate primary cells, tissues and animal models, and a more sensitive analytic technique will show whether these conclusions are also valid under real infection conditions.

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Inter-Kingdom Signaling in Bacterial Pathogenesis

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With 1 Figure

Abstract

Numerous studies have uncovered the increasing prevalence of cell-to-cell signaling as a bacterial mechanism for controlling virulence factor expression. Although there are many mechanisms for bacteria to sense environmental signals, two-component signaling systems, composed of a sensor kinase and a response regulator, are among the most common. Enterohemorrhagic *E. coli* (EHEC) senses the bacterial signal autoinducer-3 (AI-3) and the host hormones epinephrine and norepinephrine through the QseC sensor kinase to initiate regulation of virulence gene expression. QseC also activates expression of genes encoding a second two-component system (*qseEF*), where QseE, the sensor kinase, senses the stress signals epinephrine, phosphate and sulfate. Working in concert, these two two-component systems initiate a complex signaling cascade that affects both metabolism and pathogenesis.

Zusammenfassung

Zahlreiche Untersuchungen haben deutlich gemacht, dass Zell-Zell-Kommunikation zwischen bakteriellen Erregern und Wirtszellen eine wichtige Rolle für die Expression von Pathogenitätsfaktoren spielt. Trotz vielfältiger Mechanismen der Signaltransduktion werden Zweikomponentensysteme, bestehend aus Sensorkinase und Responseregulator, am häufigsten genutzt. Enterohämorrhagische *E. coli* (EHEC) erkennen den bakteriellen Autoinduktor-3 (AI-3) und die Wirtshormone Epinephrin und Norepinephrin über die Empfängerkinase QseC und regulieren über diesen Weg Virulenzgene. QseC aktiviert auch die Expressen von Genen eines zweiten Zweikomponentensystems (QseEF), wobei QseE als Empfängerkinase die Stresssignale über Epinephrin, Phosphat und Sulfat wahrnimmt. Beide Zweikomponentensysteme kontrollieren durch ihr Zusammenspiel sowohl den Metabolismus als auch die Pathogenität.

1. Introduction

Enterohemorrhagic *E. coli* (EHEC) colonizes the large bowel, causes attaching and effacing (AE) lesions and produces the potent Shiga toxin (Stx), which is responsible for the major symptoms of hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Kaper et al. 2004). The genes involved in the formation of the AE lesion are contained on a pathogenicity island named the locus of enterocyte effacement (LEE) (McDaniel et al. 1995). The LEE region contains five major operons: *LEE1*, *LEE2, LEE3*, *tir*/*LEE5* and *LEE4* (Elliott et al. 1998, 1999, Mellies et al. 1999), which encode a type III secretion system (TTSS) (Jarvis et al. 1995), an adhesin (intimin) (Jerse et al. 1990) and its receptor (Tir) (Kenny et al. 1997), and effector proteins (Elliott et al. 2001, Kanack et al. 2005, Kenny and Jepson 2000, McNamara and Donnenberg 1998, Tu et al. 2003). The LEE-encoded TTSS also translocates effector proteins encoded outside of the LEE region (NleA, B, C, D, E, F and EspFu);

these other effectors are also important for virulence and pedestal formation (Campellone et al. 2004, Dahan et al. 2005, Deng et al. 2004, Garmendia and Frankel 2005, Gruenheid et al. 2004, Mundy et al. 2004, Shaw et al. 2005). There are two types of Stx, Stx1 and Stx2, that are associated with human disease (Kaper 1998, Nataro and Kaper 1998). Neely and Friedman (1998) and Plunkett et al. (1999) demonstrated that the genes encoding Stx1 and Stx2 are located within the late genes of a λ-like phage. These genes are transcribed when the phage enters the lytic cycle (Neely and Friedman 1998), which can be triggered by the onset of an SOS response. Moreover, Kimmitt et al. (1999) reported that the induction of an SOS response in EHEC induces the production of Stx2.

EHEC senses three signals to activate its virulence genes, one is a bacterial aromatic autoinducer (AI-3) produced by the normal human gastrointestinal (GI) microbial flora, and the others are the host hormones epinephrine/norepinephrine (NE) produced by the host (Sperandio et al. 2003). Recognition of these three signals is essential for virulence expression in two different animal models (Clarke et al. 2006, Rasko et al. 2008a). AI-3 is a quorum sensing (QS) signal produced by several species of bacteria, including commensal *E. coli* as well as several other intestinal bacterial species (EPEC E2348/69, EHEC O26:H11, EPEC O111:H9, *Klebsiella pneumoniae*, *Shigella* sp., *Salmonella* sp., *Lactobacillus reuteri*, and *Enterobacter cloacae*) (Tannock et al. 2005, Walters et al. 2006). The diversity of bacteria producing AI-3 suggests that AI-3 may serve as another inter-species QS signal. Both epinephrine and NE are present in the GI tract. NE is synthesized within the adrenergic neurons within the enteric nervous system (ENS) (Furness 2000). Epinephrine is synthesized in the central nervous system (CNS) and in the adrenal medulla; it acts in a systemic manner after being released into the bloodstream, thereby reaching the intestine (Purves et al. 2001). Both hormones modulate intestinal smooth muscle contraction, submucosal blood flow, and chloride and potassium secretion in the intestine (Horger et al. 1998). Epinephrine and NE are recognized by adrenergic receptors in mammalian cells; Freddolino et al. (2004) reported that the ligand-binding sites for epinephrine and NE in a human adrenergic receptor are similar. Extensive evidence indicates that both epinephrine and NE are recognized by the same receptors and play important biological roles in the human GI tract.

AI-3 and epinephrine/NE are agonistic signals, and responses to both signals can be blocked by adrenergic antagonists (Clarke et al. 2006, Sperandio et al. 2003, Walters and Sperandio 2006). These signals are sensed by sensor kinases in the membrane of EHEC that relay this information to a complex regulatory cascade, culminating in the activation of the flagella regulon, the LEE, and Stx expression. QseC is one of these sensor kinases. QseC specifically senses AI-3/epinephrine/NE (to augment its phosphorylation state) and that QseC directly binds to NE. Thus, QseC is a bacterial adrenergic receptor. QseC's recognition of these signals can be blocked with the α-adrenergic antagonist phentolamine and the small molecule LED209, but not with the β-adrenergic antagonist propranolol (Clarke et al. 2006, Rasko et al. 2008b, Sperandio et al. 2002, 2003). QseC is essential for full virulence in EHEC, given that a *qseC* mutant is attenuated for virulence in the EHEC infant rabbit animal model (Rasko et al. 2008a). A *qseC* mutant of a rabbit enteropathogenic *E. coli* (REPEC) strain, which is a natural pathogens of rabbits, is also attenuated for disease, further confirming the important role of the QseC sensor in virulence expression (Clarke et al. 2006).

Upon sensing AI-3/epinephrine/NE, QseC phosphorylates the QseB response regulator, which activates expression of the flagella regulon (Clarke and Sperandio 2005b) and itself (Clarke and Sperandio 2005a). QseC also activates expression of the *qseEGF* genes (Rea-

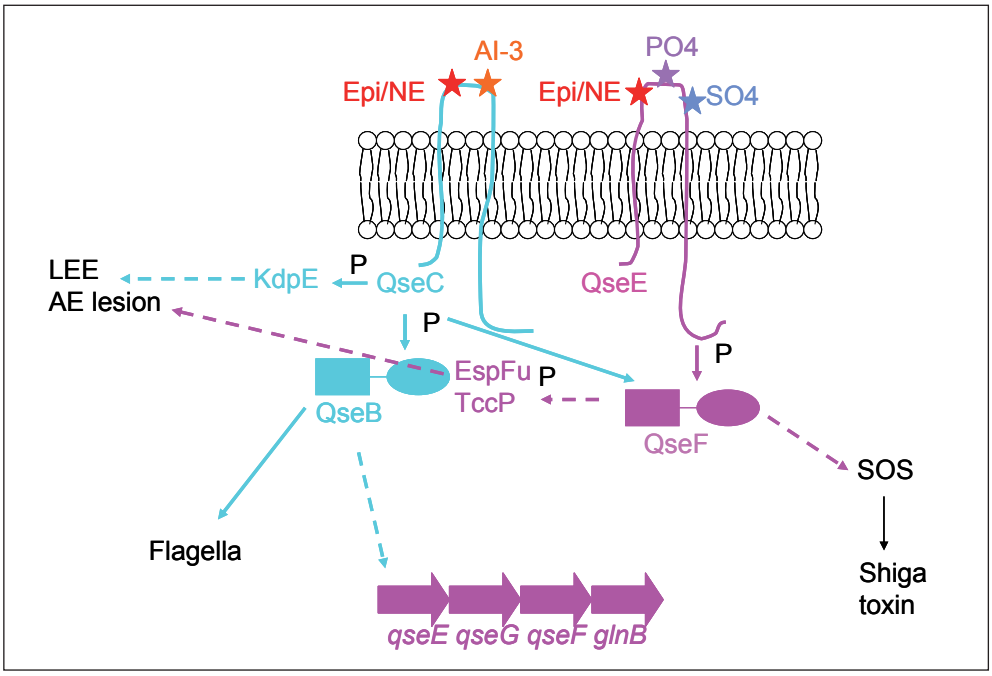

Fig. 1 QseC adrenergic signaling cascade in EHEC

DING et al. 2007), which encode a second two-component system in which QseE is the sensor kinase and the QseF the response regulator, and a lipoprotein (QseG), necessary for translocation of TTSS effectors into epithelial cells (READING et al. 2009). QseE is a second bacterial adrenergic receptor, which gages the stress signals epinephrine, sulfate and phosphate (READING et al. 2009). In addition, QseBC also plays an important role in the regulation of the LEE genes, TTS effectors encoded outside of the LEE, iron uptake systems, several adhesins, other two-component systems, and Shiga toxin. QseC can also phosphorylate other non-cognate response regulators. QseC phosphorylates QseF, which is also phosphorylated by the QseE adrenergic sensor, to coordinate expression of virulence genes involved in formation of AE lesions in the intestinal epithelia by EHEC, and the bacterial SOS stress response. The third response regulator phosphorylated by QseC is KdpE, which controls potassium uptake, osmolarity, and also the formation of AE lesions in the intestine (HUGHES et al. 2009). The fact that more than, one kinase can activate multiple response regulators suggests that there is a hierarchy of signaling, beginning with QseC. This level of control may be the fine-tuning that is observed in EHEC where the motility, formation of lesions and secretion of toxin must be exquisitely choreographed to have an effective infection occur.

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Der Band enthält 11 Vorträge aus den wissenschaftshistorischen Seminaren der Leopoldina und zeigt damit die große Themenvielfalt dieser Veranstaltungen. Behandelt werden u. a. die Problemkreise „Die Natur als Magierin: Zum paracelsischen Erbe neuzeitlicher Medizin“ (H. Schott), „Georg Ernst Stahls medizinische Theorie und der Pietismus des 18. Jahrhunderts“ (J. Helm), „Die tamilische Heilkunde in der Wahrnehmung der pietistischen Missionare der dänisch-halleschen Tranquebar-Mission in der ersten Hälfte des 18. Jahrhunderts“ (J. N. Neumann), „Matthias Jacob Schleiden und die Versammlungen Deutscher Naturforscher und Ärzte“ (I. Jahn), „Von der Adria an die Nordsee. Meeresbiologische Forschung in der Kaiser-Wilhelm-/Max-Planck-Gesellschaft“ (M. Kazemi) und „Wandel und Wende in der ostdeutschen Wissenschaft – Pflanzenbiochemie als institutionelles Beispiel“ (B. Parthier). Biographische Fragestellungen verfolgen die Beiträge „Johann von Lamont (1805–1879) – ein Pionier des Erdmagnetismus“ (H. Soffel), „Der (un)bekannte Reformer – Wilhelm Friedrich Georg Behn (1808–1878) und die Reorganisation der Leopoldina“ (M. Kaasch) und „‚Bambusstrategie‘. Max Planck in der NS-Zeit“ (E. Henning). Der Abschluss eines Bandes der Leopoldina-Ausgabe von Goethes Naturwissenschaftlichen Schriften (*Zur Farbenlehre und Optik nach 1810 und zur Tonlehre*) bildet den Hintergrund eines weiteren Referates (T. Nickol), während ethische Fragen im Fokus der Ausführungen über „Euthanasie in Geschichte und Gegenwart – im Spektrum zwischen Lebensbeendigung und Sterbebeistand“ (D. von Engelhardt) stehen. Drei Abhandlungen ergänzen den Band. Sie behandeln Leben und Wirken von Otto Meyerhof und Karl Lohmann (E. Hofmann) und widmen sich Fragen der Leopoldina-Geschichte, u. a. dem „Ende des Wanderlebens“ der einst mit den jeweiligen Präsidenten ihren Sitzort wechselnden Akademie (M. Kaasch) bzw. den Gründen für die schließlich dauerhafte Ansiedelung der Leopoldina in Halle an der Saale (W. Berg und M. Kaasch).

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From Parasitic to Endosymbiotic Lifestyles

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The Many Faces of *Photorhabdus*

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Abstract

Bacteria from the genus *Photorhabdus* have become the subject of a large number of patents in recent years due to their extensive range of insecticidal toxins and anti-microbial compounds. These are of interest to medical science and as bio-control agents for crop protection against insect pests. This enterprise-driven research has led to a better understanding of the basic biology of this genus and its mutualistic relationship with entomopathogenic nematodes (EPNs). The recent identification of a *Photorhabdus* species, which is also able to cause disease in humans, is shedding new light on the evolution of bacterial pathogens. This short review will cover the current knowledge of the lifecycles of *Photorhabdus* ssp. and their EPN symbionts.

Zusammenfassung

Bakterienarten der Gattung *Photorhabdus* haben in den vergangenen Jahren zu zahlreichen Patentierungen geführt wegen ihrer Fähigkeiten, verschiedene Insektizide und mikrobizide Substanzen zu produzieren. Diese „toxischen" Substanzen finden großes Interesse als neuartige Antibiotika zur Therapie von Infektionskrankheiten und zur Bekämpfung von Insektenplagen in der Landwirtschaft. Die durch ökonomische Interessen angetriebene Erforschung von *Photorhabdus* hat zu neuen Erklenntnissen der grundlegenden Biologie und der mutualistischen Lebensweise dieser Bakteriengattung mit entomopathogenen Nematoden (EPNs) geführt. Die kürzliche Identifizierung einer *Photorhabdus*-Art aus einem infizierten Patienten wirft ein neues Licht auf die Koevolution der Erreger-Wirtsinteraktion. Diese Kurzübersicht befasst sich mit dem Kenntnisstand der Lebenszyklen von *Photorhabdus* ssp. und seinen EPN-Symbionten.

1. The Genus Photorhabdus

Photorhabdus are Gram-negative gammaproteobacteria of the family *Enterobacteriaceae*. Currently this genus consists of three known species, *P. luminescens*, *P. temperata* and *P. asymbiotica*. Their major identifiable characteristic is the ability to form a symbiotic relationship with specific species of entomopathogenic nematodes (EPNs) from the family *Heterorhabditidae.* The lifecycles of all *Photorhabdus* ssp. revolve around the free-living infective form of their specific nematode, termed the infective juvenile (IJ). In an astounding symbiotic relationship, the nematode acts as a vector for transferring the bacteria between insect hosts. The bacteria kill and bioconvert the insect prey producing a rich nutrient source for bacterial replication. The nematode itself then replicates, whilst feeding off the abundant bacteria, before a switch back to symbiosis results in thousands of IJs emerging from the cadaver to seek out new prey.

The first *Photorhabdus* isolates were reported in 1977 and were initially classified as a new bioluminescent species of *Xenorhabdus*, termed *X. luminescens* (Khan and Brooks 1977, Poinar et al. 1977). However *Xenorhabdus* species are only known to associate with EPNs from the family *Steinernematidae.* This, along with differences in phenotypic traits and DNA sequence, led to the proposal to classify this species in a new genus, *Photorhabdus* (Boemare et al. 1993). Further characterization of individual strains led to the identification of *P. luminescens* and *P. temperata* as separate species, which have been recently been split further into subspecies (Fischer-Le Saux et al. 1999). Both *P. luminescens* and *P. temperata* are insect pathogens; however, a new species of *Photorhabdus*, *P. asymbiotica*, has been isolated from human skin lesions yet have retained their insecticidal nature (Farmer et al. 1989, Peel et al. 1999, Gerrard et al. 2004, 2006). This species represents a new stage in the evolution of this genus.

2. The Three Faces

Photorhabdus ssp. have long been known to demonstrate two very different sides to their nature. Here we have termed these the two faces of *Photorhabdus*. The first face is the mutualistic, symbiotic face which is apparent during association with the EPN. The second face is the ruthless killing machine which takes over during infection of insect larvae. These two faces are mirrored by the presence of two different phenotypic variants (PVs) of *P. luminescens* when grown in culture. Only primary PVs are able to support nematode growth, whilst both primary and secondary PVs are equally virulent to insect hosts (Forst and Clarke 2001). Other variants can occur in *Photorhabdus* resulting in unusual colony morphologies. These are termed colonial variants (CVs) and some CVs have also been demonstrated to be unable to support nematode growth (Hu and Webster 1998). The reasons behind the switch between variants are still unknown. The emergence of *P. asymbiotica* as a pathogen of both insects and humans raises the possibilty of a third face to *Photorhabdus*. Is this ability to infect a new host due to the acquisition of new virulence determinants or simply an adaptation to being able to grow at 37 ºC and utilize its pre-existing weaponry?

2.1 First Face: *Mutualistic Symbiont*

Photorhabdus and *Heterorhabditis* co-exist in a symbiotic relationship where they depend on each other in order to feed and reproduce. This relationship is extremely close and involves a multitude of, as yet unknown, signals between the two species. So close is this relationship that individual species of *Heterorhabditis* will only respond to and associate with their cognate bacterial partners (or very close relatives). Thus the specific interactions which allow bacterial retention in the nematode gut will only occur with this specific partner (Gerritsen and Smits 1997, Gerritsen et al. 1998). Both symbionts are therefore in control of the association with phenotypic changes that must occur on both sides. *Photorhabdus* produce two forms of intracellular protein crystal during stationary phase, formed by small hydrophobic proteins, CipA and CipB. These crystal are costly for the bacteria to produce, but are essential for nematode development (Bintrim and Ensign 1998, Bowen and Ensign 2001, You et al. 2006). It is also likely that *Photorhabdus* down-regulates the expression of factors, including nematicidal toxins, which it requires during host bioconversion, and begins to express adhesins and immuno-modulatory signals in order to allow it to associate with and survive in the

nematode intestinal lumen. Whilst *Heterorhabditis* must begin to express specific receptors and down-regulate its immune response in order to accept the bacterial passenger.

The death and bioconversion of the insect cadaver by *Photorhabdus* is critical for the *Heterorhabditis* lifecycle as the nematodes will only grow and develop when their cognate bacterial partners are present in high cell density and have entered their post-exponential phase (Han and Ehlers 2000). A feeding-based signal, proposed to be a biosynthetic product of a phosphopantetheinyl transferase (NrgA), is involved in this developmental regulation process (Ciche et al. 2001, Joyce et al. 2006). This strict control of nematode development is evidenced by the fact that precisely three generations of nematode replication are undergone before bacterial association occurs and the new IJs are ready to be released. The nematodes destined to become IJs start life as eggs developing inside the maternal body cavity (Ciche et al. 2008). This internal development of the larvae results in matricide, in a process known as *endotokia matricida* whereby the mother sacrifices her own life when giving birth. Maternally ingested *Photorhabdus* replicate within the intestinal lumen and invade rectal gland cells before being released into the body cavity. Here a few bacterial cells invade the pharyngeal intestinal valve cells of the developing infective juvenile and subsequently colonize the intestinal lumen (Ciche et al. 2008). Once the IJs are developed they burst free of their maternal incubator and disperse away from the insect cadaver. These new free-living IJs are soil-dwellers which actively seek out new insect larvae in order to continue the cycle. The IJ penetrates an insect host via the respiratory spiracles, mouth or anus and immediately migrates to the hemocoel (Forst and Clarke 2001). At this stage the bacteria residing in the nematode intestinal lumen are egested through the mouthparts in response to another feeding-based signal (Ciche et al. 2008).

2.2 Second Face: *Insecticidal Pathogen*

Unlike some entomopathogenic species, *Heterorhabditid* nematodes and their associated *Photorhabdus* are both generalists when it comes to the insect species which they prey upon. A variety of insect orders including Lepidoptera, Coleoptera, Hymenoptera and Dictyoptera are susceptible to the full reproductive cycles of the nematode and bacterial symbionts (Bowen and Ensign 1998). Some symbionts have also been shown to be pathogenic to non-insect species such as isopods, however, these appear to be a reproductive dead-end as no infective juveniles are able to be generated indicating that specific cues from the insect itself are required (Sicard et al. 2008). However, these reproductive dead-ends may just be the beginnings of a new phase in the evolution of the symbiotic pairing.

Laboratory-based infection models of *Photorhabdus* usually involve *Manduca sexta* as the insect host and these have been used to great effect to reveal the development of *Photorhabdus* infection and the mechanisms it uses to evade the host immunity. Bacteria are released directly into the hemocoel where they immediately encounter the humoral (microbial recognition proteins, anti-microbial peptides, melanization) and cellular (phagocytic hemocytes) immune responses. In order to survive, *Photorhabdus* encodes a variety of mechanisms to counter these responses, however, a prior infection with non-pathogenic *Escherichia coli* appears to elicit a temporary immunity against *P. luminescens* (Eleftherianos et al. 2006). This is due to a temporary up-regulation of the humoral response indicating that at this stage it is a fine line between a successful infection or clearance of the bacteria. Several *Photorhabdus* genes (*phoP* and *pbgPE*) have been implicated in resistance to the anti-microbial peptides and in particular, a serralysin-type protease, PrtA, has been found to target proteins

with immune related function in *in vitro* assays (Derzelle et al. 2004, Marokházi et al. 2004, Bennett and Clarke 2005, Felföldi et al. 2009). Immune responses in insects are often characterized by a rapid darkening due to the synthesis of insoluble melanin which isolates the invading pathogens. This response is controlled by the prophenoloxidase cascade, which in turn is regulated by the eicosanoid pathway which also controls the formation of hemocyte aggregates known as nodules (Lavine and Strand 2002). An interesting feature of *Photorhabdus* infection is the noticeable lack of melanization due to specific inhibition of phospholipase A_2 (PLA_2) which regulates the eicosanoid pathway and prophenoloxidase cascade (ffrench-Constant et al. 2003, Kim et al. 2005, Eleftherianos et al. 2007, 2009). Other mechanisms of immune evasion specifically target the insect hemocytes in order to prevent phagocytosis. All *Photorhabdus* ssp. encode a Type III secretion system which is able to inject immunomodulatory effector proteins directly into the phagocytic hemocytes (Brugirard-Ricaud et al. 2004, 2005). A Type III effector protein from the Cif family of proteins has been identified which is able to arrest the cell cycle of the cultured human HeLa cell line and may have similar effects on insect hemocytes (Jubelin et al. 2009, Yao et al. 2009). Interestingly, unlike the other *Photorhabdus* species, *P. asymbiotica* may actively encourage phagocytosis and has actually been found replicating within hemocytes from the lepidoteran *Mythimna unipuncta* (Costa et al. 2009).

Bacteria which are able to survive the immune responses in the hemocoel rapidly begin to colonize the anterior of the midgut and subsequently spread along its length (Silva et al. 2002). Colonization appears to be restricted to folds between the extracellular matrix and the basal side of the midgut epithelium. Once colonization has been achieved, the co-ordinated secretion of insecticidal toxins results in the destruction of the midgut epithelium and cessation in feeding behavior ending in starvation. These specific toxins are the focus of much of the enterprise-driven research into *Photorhabdus* as a resource for bio-control agents. Several, including the toxin complexes (Tc's) and the binary toxins PirAB, have been patented for use either for crop protection or targeted removal of insect disease vectors. Almost all have an unknown mode of action and an unknown specificity which makes their use controversial without further rigorous testing, especially in crops destined for human consumption.

After the death of the insect host, bacteria spread throughout the cadaver and begin bioconversion of the tissues. This generates a rich nutrient soup for bacterial growth, which in turn leads to a plentiful food source for the nematodes (ffrench-Constant et al. 2003). During bioconversion, *Photorhabdus* ssp. produce a wide variety of small molecule antibiotics and anti-fungals, as well as molecules which repel foraging insects, thus protecting the cadaver and ensuring the hard-earned nutrients are not stolen by opportunists (Baur et al. 1998, Hu and Webster 2000, ffrench-Constant et al. 2000, Derzelle et al. 2002, Zhou et al. 2002, Hu et al. 2006, Bode 2009).

2.3 Third Face: *Emerging Human Pathogen*

The ability of *P. asymbiotica* to cause local soft tissue infections in humans is an interesting step in the evolution of this genus. Firstly, it has overcome an inability to grow at 37 ºC which is evident in the other *Photorhabdus* species. Secondly, it has also evolved mechanisms to evade the human immune system and to spread from the original site of infection. These newly acquired abilities and the mechanisms behind them are only just now being understood, mainly due to the recent sequencing of the genomes of *P. asymbiotica* and *P. luminescens* strains (Duchaud

et al. 2003, Wilkinson et al. 2009). The most obvious difference between the two species is the loss or truncation of many of the insecticidal toxin loci in *P. asymbiotica*. This would imply a change in lifestyle away from using insects as a host resulting in a reduction in the insect specific virulence factors. However, in laboratory models, *P. asymbiotica* is actually more virulent to insects than *P. luminescens* (Wilkinson et al. 2009). Therefore, this may actually indicate a switch in virulence mechanisms to a more effective strategy which does not require the use of these toxins. Potentially these new mechanisms may work equally well against insect and human immune cells and it is this switch which has allowed human infection to occur. One of these new mechanisms is the ability to survive inside phagocytic cells (Costa et al. 2009). The other *Photorhabdus* species specifically inhibit phagocytosis by insect hemocytes, whereas *P. asymbiotica* have been found replicating within the hemocytes of the Lepidoteran *Mythimna unipuncta* after infection. Similarly *P. asymbiotica* is also able to survive and replicate within human macrophages, and strains from Australia have even been shown to invade non-phagocytic human cell lines (Costa et al. 2009). It is therefore likely that the presentation of abscesses at multiple sites after initial infection is due to the bacterium hitching a ride in immune cells such as macrophages. Analysis of the *P. asymbiotica* genome has revealed the presence of a second Type III secretion system (T3SS2), similar to the situation in other facultative intracellular human pathogens such as *Yersinia* and *Salmonella* ssp. T3SS2 is associated with an orthologue of the *Salmonella* effector SopB which may be involved in controlling the maturation of vacuoles containing internalized bacteria (Bakowski et al. 2008).

Strangely, *P. asymbiotica* has successfully evolved into a human pathogen, yet this does not appear to be a beneficial step for its symbiont, *H. gerrardi*. The symbiotic pairing has been isolated from the soil at the site of human infections, but the nematode cannot replicate in the human host and thus the mutualistic relationship breaks down. Perhaps the ability to infect humans is purely a side effect of an increased virulence towards insects, or perhaps the symbiotic pairing can successfully infect small mammals and both complete their lifecycles. So far there is no evidence for this, but this may be simply because no one has looked for *Photorhabdus* in the cadavers of small mammals.

3. Conclusions

The genus *Photorhabdus* has evolved into a highly efficient insect killer and yet manages to balance this with a mutualistic co-operative attitude towards its cognate nematode partners. These two opposite sides to its nature require precise control over gene expression in order to achieve such a fine balance between them. However *P. asymbiotica* appears to have begun to tip that balance towards the pathogenic side with the acquisition of factors which enable it to infect new hosts with no regard for whether its symbiont can successfully reproduce within them. Thus *P. asymbiotica* represents not only an emerging human pathogen, but also a break from the symbiotic cycle.

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Functional Analysis of *Blochmannia floridanus*, the Primary Endosymbiont of the Carpenter Ant *Camponotus floridanus*

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Abstract

Insect-prokaryote symbioses are widespread and usually compensate for the hosts' unbalanced nutrition. Carpenter ants (genus *Camponotus*) are omnivorous animals, but nevertheless harbor endosymbiotic bacteria (genus *Blochmannia*). Antibiotic feeding experiments with a defined diet confirmed the importance of essential amino acid synthesis by the bacteria for the colony's breeding success. Yet, tracking of symbiont location and density during the ants' development suggests that this symbiosis has a temporal relevance, especially during the pupal phase, when the animals rely on internal storage. Bacterial gene regulation during host development is modest, but at the end of metamorphosis expression of symbiosis factors peak compared to the genes of basic metabolism. In the adult stages of the host, *Blochmannia* is removed from the ants' guts, pointing to a domestication of the bacteria by the host.

Zusammenfassung

Symbiosen zwischen Insekten und Prokaryoten sind weit verbreitet und kompensieren in der Regel unausgewogene Nahrungsquellen der Wirtstiere. Rossameisen (Genus *Camponotus*) weisen eine omnivore Ernährungsweise auf, beherbergen jedoch ebenfalls endosymbiontische Bakterien (Genus *Blochmannia*). Fütterungsversuche mit Antibiotika und einer definierten Diät bestätigten eine Relevanz der Bakterien für den Bruterfolg der Ameisenkolonie durch Biosynthese von essentiellen Aminosäuren. Variationen in der Lokalisation und Anzahl der Symbionten während der Entwicklung der Wirtstiere deuten auf eine zeitlich begrenzte Bedeutung der Symbiose hin, besonders während der Puppenstadien, in denen die Tiere vollständig auf interne Reserven angewiesen sind. Die Endosymbionten zeigen nur ein geringes Ausmaß an Genregulation, jedoch weisen symbioserelevante Gene gegen Ende der Metamorphose verglichen mit Genen des bakteriellen Grundmetabolismus eine erhöhte Expression auf. Adulte Wirtsstadien besitzen eine deutlich reduzierte Symbiontenanzahl, wodurch eine Domestikation der Bakterien durch den Wirt wahrscheinlicher ist als eine rein mutualistische Beziehung.

1. Introduction

Insects constitute an evolutionary group that has undergone enormous diversification and that is present in virtually every terrestrial habitat in great numbers, both individual and species (Mayhew 2007). One explanation for this success is the widespread presence of bacterial endosymbionts (Buchner 1965, Douglas 1989, Moran and Telang 1998) which allow insects to survive in niches that otherwise would be unsuitable to them, mostly due to unbalanced diets

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(Baumann et al. 2006, Feldhaar and Gross 2008). The symbionts, in general, encode metabolic pathways complementary to the hosts' needs. *Buchnera aphidicola*, the primary symbiont of aphids, is able to synthesize most essential amino acids (Shigenobu et al. 2000, Moran and Degnan 2006) as its host completely relies on plant sap that is rich in carbohydrates but lacking in essential amino acids (Douglas 2006). Tsetse flies, on the other hand, feed on mammalian blood lacking vitamins and cofactors, which are provided by its primary endosymbiont *Wigglesworthia glossinidia* (Akman et al. 2002). Ants of the genus *Camponotus,* and other closely related ant genera, harbor bacterial endosymbionts of the genus *Blochmannia* in specialized midgut cells called bacteriocytes (Blochmann 1882, Buchner 1965, Sauer et al. 2000, 2002). In contrast to the majority of insect hosts of primary endosymbionts, *Camponotus* ants are nutritional generalists in many terrestrial habitats (Hölldobler and Wilson 1990, Bolton 1995, Pfeiffer and Linsenmair 2000), therefore, the relevance of *Blochmannia* is not obvious at first sight. Analysis of the genome sequences of *B. floridanus* and *B. pennsylvanicus* revealed, as in most primary endosymbionts, dramatically reduced genome sizes of 705 kb and 792 kb with extreme A/T contents of over 70 % (Gil et al. 2003, Degnan et al. 2005). Genes that have become obsolete in the stable intracellular environment have been lost, including most factors with regulatory functions. Among the genes that were retained during the reductive genome evolution are pathways leading to all essential amino acids as well as other putative symbiotic functions such as nitrogen recycling via a bacterial urease, sulfate transport and reduction of fatty acid biosynthesis, indicating a general role of the symbionts in the hosts' nutrition.

2. Nutritional Upgrading for a Generalist?

Although the occurrence of *Blochmannia* in all *Camponotus* species examined so far suggests an obligate and important role of this symbiosis; curing adult workers of their bacteria by antibiotics does not significantly affect their mortality. Yet, at the colony level these ants show a reduced success in raising brood (Zientz et al. 2006). The number of symbionts per host is correlated with the developmental stage of the animals with highest symbiont density in late pupae and freshly hatched workers, and strongly decreased bacterial numbers in older workers (Wolschin et al. 2004, Stoll et al. 2009b). This suggests a temporal importance of the symbiosis, especially at times when the host is reliant on its internal storage during metamorphosis. Feeding experiments with chemically defined diets combined with antibiotic treatment have shown that the reduced breeding success of aposymbiotic colonies can be compensated by supplementing essential amino acids and that a lack of these substances in the host's nutrition can be compensated by *Blochmannia* in untreated colonies (Feldhaar et al. 2007).

3. Symbiont Location During Host Development

Previous studies have shown that in larvae and adult stages *Blochmannia* resides in bacteriocytes that are intercalated between the midgut cells (Buchner 1965, Sauer et al. 2002). By fluorescent *in situ* hybridization the location of the endosymbionts could be tracked during the complete life cycle of the host including metamorphosis, which might be the decisive stage for this symbiosis (Stoll 2009). In late embryonal stages the future midgut is surrounded by a layer of densely filled bacteriocytes, which is torn apart by the growth of the uninfected gut cells

during larval development. Soon after pupation, the bacteriocytes seem to proliferate, again surrounding the midgut. In addition, normal gut cells are now infected by *Blochmannia* in a yet unknown manner, leading to a heavy infection of the complete midgut at the end of metamorphosis with only a few cells remaining uninfected. In adult animals the symbiosis degenerates and symbiont numbers decrease with the increasing age of the workers. Bacteria are then restricted to original bacteriocytes (Stoll 2009). Possibly auto-/xenophagy (Levine 2005) in the newly infected cells via the host's lysosomal system represents an additional way of endosymbiont number control and of direct transport of symbiotic metabolites to the host during times of need as observed in the aphid – *Buchnera* symbiosis (Nishikori et al. 2009).

4. Bacterial Gene Expression during Host Development

The detected dynamic distribution of *Blochmannia* during the development of its host raised the question whether the symbionts are still able to adapt to changing external conditions despite the small number of transcriptional regulators annotated in the genome sequence (Gil et al. 2003, Degnan et al. 2005). The transcriptome of *B. floridanus* was assessed during eight representative developmental host stages and modest regulatory effects were observed during the host's life cycle with changes in gene expression rarely exceeding factor three (Stoll et al. 2009b). The expression profiles of genes located next to each other on the same DNA strand are often similar, suggesting the presence of local transcription units. Although *in silico* predictions of vegetative promoters fail due to the extreme A/T content of the genome, mapping of 5' ends of several *B. floridanus* mRNAs indicated transcription initiation at distinct and conserved sites (Stoll et al. 2009a). Four operons in *B. floridanus* are dependent on the only alternative sigmafactor RpoH, which mainly regulates heat shock response in *E. coli* (Arsène et al. 2000). Yet most *B. floridanus* genes under the control of RpoH are already strongly expressed under vegetative conditions and are not significantly inducible with heat treatment (Stoll et al. 2009a, b). *Blochmannia* also seems to possess mechanisms of global gene regulation, as genes belonging to related pathways often share similar expression profiles over the different host stages (Stoll et al. 2009b). Transcription, especially of genes of the basic bacterial metabolism and genes with putative symbiotic functions, appears to be uncoupled, with symbiotic factors being most strongly expressed at the end of the pupal phase. Regarding absolute levels of transcription, aside from many putative symbiotic factors, classic stress-related genes such as those encoding the molecular chaperons GroES/EL, DnaK/J are especially strongly expressed. This has also been reported for other primary endosymbionts (Ishikawa 1984, Haines et al. 2002, Wilcox et al. 2003, Fares et al. 2004, Viñuelas et al. 2007) and is possibly an adaption to the fast evolutionary rate of these symbionts due to missing DNA repair mechanisms to rescue misfolded proteins (Huang et al. 2008). In line with this phenomenon, strongly expressed (and therefore essential) genes accumulate less mutations and retain a higher G/C content (Schaber et al. 2005, Viñuelas et al. 2007, Stoll et al. 2009b).

5. Mutualism versus Domestication

The recent insights into the symbiosis between *Camponotus* and *Blochmannia* shed a new light on the classical view of a relationship with mutual benefits for both partners and help

to explain the riddle of which requirements a generalist may have in a nutrition-based symbiosis. *Blochmannia* can be seen as a production unit, streamlined by evolution for the production of essential nutrients, while the host is adjusting symbiont numbers according to its needs. The residual regulatory capacities of the symbiont may represent a mechanism of fine tuning to the changing conditions in the host cell, but when the costs of maintaining the symbionts outweigh the benefits for the host in adult animals, the bacteria are removed. The prime importance of this symbiosis seems to be the supply of high quality nutrients during a period when the host is completely reliant on its internal storage, which deplete with high metabolic rates during metamorphosis. As only successful ant colonies will transfer their symbiont lineages to the next generations, the bacteria are forced to suffer this domestication for the benefit of the ants.

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Neue Bilder vom Alter(n)
Wettbewerb und Ausstellung

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Die Menschen leben länger, und der Anteil alter Menschen in der Gesellschaft nimmt zu – das macht den demographischen Wandel zu einem brisanten gesellschaftlichen Thema, denn „Altern" berührt in besonderer Weise das Leben jedes Einzelnen und unsere Gesellschaft, ihren Wohlstand und ihre Verfasstheit. Die Leopoldina hat sich daher über einen längeren Zeitraum mit dem „Altern in Deutschland" beschäftigt. Das Altersbild und die verfügbaren Alternsrollen sind vielfach noch durch eine traditionelle Sichtweise geprägt. Die anstehenden Veränderungen erfordern jedoch einen veränderten Blickwinkel. Der Katalog zeigt Bilder des Alterns, die sich den neuen Herausforderungen und Potentialen des Älterwerdens des Einzelnen und der Gesellschaft auf verschiedene Weise stellen. Er ergänzt damit im Dialog mit der Gesellschaft die wissenschaftlichen Veröffentlichungen der Akademie zur Alternsproblematik um einen künstlerischen Bezug.

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Stages of Metabolic Adaptation of Endosymbiotic Bacteria

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With 2 Figures and 1 Table

Abstract

A model to explain the evolutionary history of animal-bacteria obligatory mutualistic symbiosis is presented. Dispensability of genes and adaptability to the host are key factors in the reduction process of these bacterial genomes. Major steps in such genome reductive evolution, leading towards primary endosymbiosis, and the possibility of complementation or replacement by a secondary symbiont are also indicated. Yet, we need to understand what happens at the beginning of the adaptive process towards an obligate mutualistic relationship.

Zusammenfassung

Ein Modell, das die Evolutionsgeschichte von obligaten mutualistischen Symbiosen zwischen Tieren und Bakterien erklärt, wird präsentiert. Entbehrlichkeit von Genen und Anpassungsvermögen an den Wirt sind Schlüsselfaktoren in dem Reduktionsprozess dieser Bakteriengenome. Entscheidende Schritte einer solchen reduktiven Evolution, die zu primärer Endosymbiose führt, und die Möglichkeit von Komplementation oder Ersetzung durch einen sekundären Symbionten werden ebenso aufgezeigt. Immer noch unverstanden ist allerdings die initiale Phase des adaptiven Prozesses hin zu obligaten mutualistischen Beziehungen.

1. Several Orders of Bacteria and Archaea Contain Coherently Reduced Genomes

Genome reduction is a phenomenon often correlated with the evolution of an intracellular lifestyle, i.e. the stable and permanent integration within a cell that is usually eukaryotic (Moran et al. 2008, 2009). Numerous symbionts, parasites, and pathogens have developed such lifestyles (Buchner 1965, Baumann 2005, Casadevall 2008). The trigger for the reduction of the genome is the permanent and irreversible incorporation of the bacterial (or archaeal) cell into the body of the eucaryotic organism, which is from then on the host. The embedding in a stable cellular environment and the provision with an amplitude of metabolites that is possible within the eucaryotic body allows the intracellular bacteria to lose major parts of their metabolic and also regulatory capacities (Silva et al. 2001, 2003, Darby et al. 2007). This economization leads to compact genomes that usually are only 10 – 30 % of the size of their closest free-living relatives.

Endosymbiosis and the associated evolution of reduced genomes has occurred independently in various groups. An analysis of the phylogenetic identity of these intracellular organisms reveals that this lifestyle is present in a wide range of different taxa. Figure 1 shows

an analysis of the two main genetic parameters, genome size and mol% G+C content of the DNA for a variety of bacteria highlighting two groups, Bacteroidetes and Enterobacteriales, representing two different phyla. As can be seen both groups span a comparable range of gene numbers (a parameter that is closely correlated to genome size), both regarding symbionts and free-living organisms. As regards G+C content, the Enterobacteriales as gamma-proteobacteria have generally higher values than members of the Bacteroidetes. Among the symbionts no difference is visible between the two groups, although a clear indication is difficult since, to date, only two symbionts belonging to the Bacteroidetes have been sequenced.

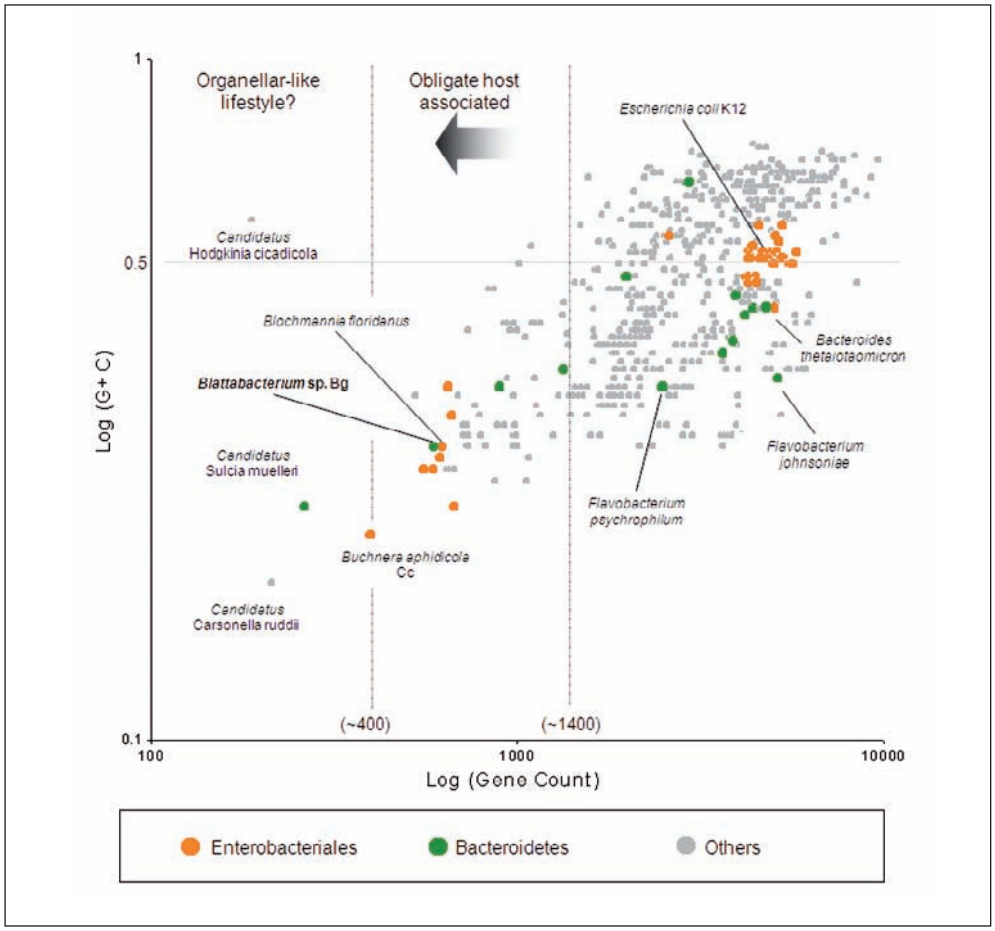

Fig. 1 The evolution of reduced gene contents. The number of genes versus the proportion of G+C is shown for genomes from Bacteria and Archaea with complete genome sequence available in public databases. The location of genomes belonging to Enterobacteriales and Bacteroidetes is emphasized. The vertical bars indicate the position along the x axis, of a) the free-living prokaryote with the smallest gene content (the heterotrophic uncultured HTCC2181 bacterium from the order Methylophilales, with 1377 genes) and b) for the smallest endosymbiotic bacterium (*Buchnera aphidicola* Cc with 397 genes). Although there is no direct evidence, the severe lack of several important genes in prokaryotes with smaller gene contents, suggest that they require the import of proteins coded in the genome of their host into their own cytoplasm for survival, therefore acquiring the status of organelles.

Tab. 1 Phylogenetic distribution and basic genetic characters of obligatory intracellular living bacteria with completed genomes.

Phylum/Class	Symbiont (Genus)	Lifestyle[1]	Host[2]	# of sequenced genomes[3]	range genome sizes[3]	range %G+C[3]
Proteobacteria gamma	*Buchnera*	S	I	6	0.42–0.66	20.2–26.4
	"*Candidatus* Blochmannia"	S	I	2	0.71–0.79	27.4–29.6
	"*Candidatus* Baumannia"	S	I	1	0.69	33.2
	"*Candidatus* Carsonella"	S	I	1	0.16	16.6
	Sodalis	S	I	1	4.29	54.5
	Wigglesworthia	S	I	1	0.70	22.5
	"*Candidatus* Ruthia"	S	B	1	1.20	34.0
	"*Candidatus* Vesicomyosocius"	S	B	1	1.00	31.6
	Vibrio[4]	S	F	2	4.25–4.48	38.2–38.3
	Photorhabdus[4]	S	N	1	5.69	42.8
Proteobacteria alpha	*Anaplasma*	P	M	3	1.2–1.47	41.6–49.8
	Bartonella	P	M	5	1.4–2.62	38.2–38.8
	Brucella	P	M	9	3.29–3.31	57.2–57.3
	Ehrlichia	P	M	5	1.18–1.51	27.5–30.1
	"*Candidatus* Hodgkinia"	S	I	1	0.14	58.4
	Neorickettsia	P	M	1	0.86	41.0
	Orientia	P	M	2	2.0–2.1	30.5
	Rickettsia	P	M	12	1.11–1.59	28.9–32.6
	Wolbachia	P S	I N	4	1.08–1.59	34.2–35.2
Bacteroidetes Flavobacteria	*Blattabacterium*	S	I	1	0.64	27.1
	"*Candidatus* Sulcia"	S	I	1	0.25	22.4
	"*Candidatus* Amoebophilus"	S	Pr	1	1.9	35.0
	"*Candidatus* Azobacteroides"	S	Pr	1	1.21	32.9

Phylum/Class	Symbiont (Genus)	Lifestyle[1]	Host[2]	# of sequenced genomes[3]	range genome sizes[3]	range %G+C[3]
Chlamydiae	*Chlamydia*	P	M	7	1.0–1.08	40.3–41.3
Chlamydiae	*Chlamydophila*	P	M	7	1.14–1.23	39.2–40.6
	"*Candidatus* Protochlamydia"	P	Pr	1	2.41	34.7
"Elusimicrobia"	*Elusimicrobium*	S	Pr	1	1.6	40.0
	uncultured Termite group 1 bacterium phylotype Rs-D17	S	Pr	1	1.12	35.2
Firmicutes	*Mycoplasma*	P	M	15	0.58–1.36	23.8–40.0
Mollicutes	*Mesoplasma*	P	PI	1	0.79	27.0
	Phytoplasma	P	PI	2	0.6–0.86	21.4–27.7
	Ureaplasma	P	M	3	0.75–0.87	25.5–25.8

[1] S: symbiotic, P: intracellular pathogenic/parasitic. [2] B: bivalves, F: fish, I: insects, M: mammals, N: nematodes, Pr: protozoa, Pl: plants. [3] Data taken from NCBI. [4] Free-living members of the genus known.

The majority of known intracellular bacteria belong to the proteobacteria. Therein, the largest number is gamma-proteobacteria and then alpha-proteobacteria (Tab. 1). Additionally, in the phyla Bacteroidetes and Firmicutes intracellular organisms are also found, however, in the latter phylum no symbiotic representative is known.

Two other genomic features that usually are in parallel with the size reduction developed by intracellular bacteria are an increase in A+T content of the DNA and accelerated mutation rates leading to higher numbers of deleterious mutations (Moran 1996, Silva et al. 2001).

2. Endosymbiosis is a Widespread Phenomenon

Symbiosis in the narrower sense of mutual symbiosis is a frequent phenomenon in nature especially affecting a huge diversity of Bacteria and Eucarya (Moya et al. 2008). Several groups have a multitude of representatives living in symbiosis (Fig. 2). The phylum proteobacteria contains the highest number of symbionts among the Bacteria. A more recently (re-)discovered group of bacterial symbionts are the ones pertaining to the phylum Bacteroidetes. In 1931 Hollande and Favre described the symbiont of cockroaches as new genus *Blattabacterium* (Dasch et al. 1984). These were later identified as members of the phylum Bacteroidetes, class Flavobacteria, and therefore set in opposition to the until then described proteobacterial symbionts (Bandi et al. 1994). In the last few years, additionally, another symbiont inside this group was described, "*Cand.* Sulcia muelleri", symbiont of the sharpshooter *Homalodisca coagulata*. Interestingly, *Blattabacterium* spp. and "*Cand.* S. muelleri" form together with organisms found in ladybird beetles a monophyletic group of insect-associated organisms that constitute an independent lineage within the class Flavobacteria (López-Sánchez et al. 2008).

Apart from these two groups many other bacterial phyla contain representatives that live as symbionts (Moya et al. 2008) that are associated with an extremly wide variety of diverse eukaryotic organisms, especially animals, plants and fungi (Fig. 2). The various pairs of symbiotic partners, host and symbiont, result in a wide array of different functional associations.

3. Bacterial Symbiosis in Insects

Insects, together with the Porifera, are the group of Metazoa (Moya et al. 2008) in which the highest number of bacterial symbionts was found (Fig. 2). A number of insect symbioses is well studied and understood, particularly the ones between *Buchnera aphidicola* and aphids, "*Cand.* Blochmannia spp." and carpenter ants, or *Wigglesworthia glossinidia* and tsetse fly.

The symbioses are generally based on a contribution of the symbiont to the diet of the host thereby supplementing deficiencies that exist in the natural diets of the hosts, such as phloem, xylem, or blood. This is the case of *B. aphidicola,* "*Cand.* Baumannia cicadellinicola", or *Wigglesworthia glossinidia*, respectively (Akman et al. 2002, Latorre et al. 2003, Wu et al. 2006). Symbioses are usually of mutual character, interchanging the stable environment inside the host cell for the provision with nutrients or other metabolic advantages (Moran et al. 2008, Moya et al. 2008, Hongoh et al. 2008). However, not all associations are obligatory like the ones mentioned above where the symbiont is called primary. Other symbioses have a facultative character, as is the case e.g. for "*Candidatus* Hamiltonella defensa" (Moran et al. 2005) and the respective organisms are called secondary symbionts.

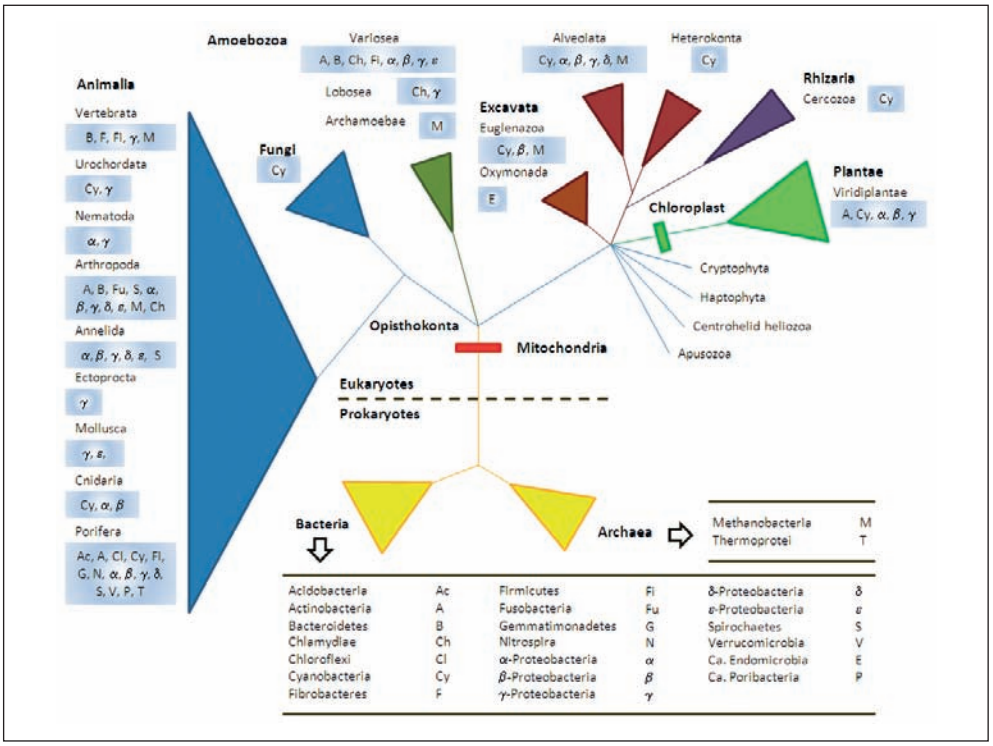

Fig. 2 Phylogenetic distribution of bacterial and archaeal symbionts. Several lineages of eukaryotes have evolved stable symbiosis with members of different classes of Bacteria and Archaea independently. Examples of ancient evolutionary symbioses are the mitochondrial respiratory chain and oxygenic photosynthesis in the chloroplast. Many more symbiotic associations have evolved more recently or are in the early stages of developing. The well studied symbiotic relationships between intracellular bacteria and insects have a proven biochemical foundation, where one of the partners benefits from compounds that are produced by the other. The figure is modified from Moya et al. 2008. The branches are not to scale.

In the recent years evidence accumulated that the association between host and symbiont has a dynamic character, and the type of association changes during the course of evolution. This will be discussed in the following sections.

3.1 Young Symbiosis

If since the establishment of the symbiosis not more than 20 Mya have passed, the process of genome reduction is still in its beginning and the size of the genome of the symbiont still largely resembles that of free-living relatives (Silva et al. 2001). Well-known examples for a young symbiosis are the associations between beetles of the genus *Sitophilus* and their primary endosymbionts, SOPE and SZPE (*S. oryzae* and *S. zeamais* PE, respectively; Heddi et al. 1999, Gil et al. 2008). In this state of symbiotic association the endosymbiont still possesses a large genome. The typical reduction process has only begun and genome sizes resemble still that of free-living relatives. Other examples for symbioses in its initial state are those where the association has only facultative character as it is the case for secondary endosym-

bionts. Examples are *Hamiltonella defensa*, *Arsenophonus* sp., and *Serratia symbiotica* that are facultative associated with aphids or white flies (Thao and Baumann 2004, Lamelas et al. 2008, Degnan et al. 2009). In general, they have genome sizes of at least 2 Mb. Evidence from sequencing shows that in this initial phase of loose association the genome contains large amounts of mobile elements such as insertion sequences or phage-related DNA (Gil et al. 2008, Degnan et al. 2009).

3.2 Advanced Symbiosis

Advanced symbioses are characterized by a progressed genome reduction of the symbiont like it is found e.g. in *Buchnera aphidicola*. The symbionts are primary symbionts of their respective hosts. Genome sizes are typically between 600 and 800 kb (Tab. 1). Due to the long period of shared evolutionary history the symbiont is well adapted to the host. This is usually expressed in a tightly expressed biochemical basis of the symbiosis as it, is the supply of amino acids in the case of *B. aphidicola*, of nitrogen and sulfur compounds by "*Cand.* Blochmannia", or of cofactors by *W. glossinidia*. Various genera contain members that fall in this group: *Buchnera,* "*Cand.* Blochmannia", "*Cand.* Baumannia", *Wigglesworthia*, *Wolbachia*, "*Cand.* Sulcia", and *Blattabacterium* (Moya et al. 2008, López-Sánchez et al. 2009). For *Portiera aleyrodidarum*, primary symbiont of the white fly *Bemisia tabaci*, the genome size is not yet determined; based on mol% of the G+C content, a reduced size of 600 – 700 kb can be estimated.

3.3 Replacement and Coexistence

In some cases very advanced states of symbiotic associations have been described (Pérez-Brocal et al. 2006, Nakabachi et al. 2006). The symbiont *B. aphidicola* BCc of the cedar aphid *Cinara cedri* has a genome of only 422 kb (Pérez-Brocal et al. 2006). This primary endosymbiont is accompanied by another, secondary symbiont, *Serratia symbiotica* (Moran et al. 2005). Together they have developed a system of metabolic complementation that is expressed in a shared pathway for tryptophan biosynthesis (Gosalbes et al. 2008). The loss of certain essential genes like for the replication or DNA repair machinery in the primary symbiont BCc raises the question to what degree the evolutionary reduction process has advanced so that in the future the symbiont might lose independence and evolve into an organelle-like body as described in other cases (Nakabachi et al. 2006, Tamames et al. 2007). The presence of the secondary symbiont can be interpreted as the initiation of a possible upcoming replacement (Moya et al. 2009). Another example for complementation is found among the cosymbionts of the sharpshooter *H. coagulata*, namely "*Cand.* B. cicadellinicola" and "*Cand.* S. muelleri" (Wu et al. 2006). The former gamma-proteobacterium produces cofactors and vitamins whereas the flavobacterial symbiont has the potential for the synthesis of amino acids (Wu et al. 2006, McCutcheon and Moran 2007).

4. Evolutionary Convergence

Different organisms of unrelated lineages can develop, along the course of evolution, the same biological characteristics. As an example for convergence, genome analysis of the en-

dosymbionts of cockroaches, *Blattabacterium* sp., revealed that these flavobacteria retained a gene set comparable to that of "*Cand.* Blochmannia spp. (López-Sánchez et al. 2009). The two types of symbionts are phylogenetically unrelated, belonging to the phyla Bacteroidetes and Proteobacteria, respectively. However, their hosts share a basically omnivorous way of feeding. An analysis of the distribution of the genes for their functional categories along the COG classification showed that the two bacteria share significantly more similar profiles of gene sets than they share with respective phylogenetically closer related symbionts such as "*Cand.* Sulcia muelleri" and *Buchnera aphidicola*, or free-living relatives such as *Flavobacterium psychrophilum* and *E. coli*, respectively.

A specific coincidence in both symbionts, blattabacteria and *Blochmannia* spp., is the presence of a urease. This catabolic enzyme is not frequently present among bacteria and among endosymbionts. It is found only in these two organisms, *Vibrio fischeri* and *Photorhabdus luminescens*, gamma-proteobacterial symbionts of marine animals and nematodes, respectively, and additionally in the intracellular pathogen, *Ureaplasma parvum*, belonging to the Mollicutes.

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