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Escherichia coli – Facets of a Versatile Pathogen

On the Occasion of the 150th Birthday of Theodor Escherich (1857–1911)

Leopoldina Symposium
Kloster Banz, Bad Staffelstein, October 9 to 12, 2007

Gabriele Blum-Oehler, Ulrich Dobrindt, Jörg Hacker, and Volker ter Meulen (Eds.)

Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2008
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HARALD ZUR HAUSEN

Vizepräsident der Akademie

NEUE FOLGE NUMMER 359 BAND 98

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Deutsche Akademie der Naturforscher Leopoldina
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European Molecular Biology Organization (EMBO)
and the
Federation of European Microbiological Societies (FEMS)

Bildungszentrum Kloster Banz, Bad Staffelstein, Germany
October 9 to 12, 2007

Editors:

Gabriele BLUM-OEHLER (Würzburg)

Ulrich DOBRINDT (Würzburg)

Jörg HACKER (Würzburg – Berlin)
Senator of the Academy

Volker TER MEULEN (Würzburg – Halle/Saale)
President of the Academy

With 22 Figures and 7 Tables

Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 2008
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Scanning Electron Microscope image of an *Escherichia coli* biofilm from a continuous flow culture; magnification 5.000 × (Author: Dr. Jaroslaw Zdziarski, Institut für Molekulare Infektionsbiologie, Röntgenring 11, 97070 Würzburg, Germany; Author, Meeting's logo: Hilde Merkert, Institut für Molekulare Infektionsbiologie, Röntgenring 11, 97070 Würzburg, Germany).

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Welcome Address of the German Academy of Sciences Leopoldina

Hubert E. Blum ML (Freiburg)

Member of the Academy Presidium

Dear Colleagues,
Ladies and Gentlemen,

I would like to welcome you on behalf of the German Academy of Sciences Leopoldina, co-sponsor of this symposium, and its president Professor Volker ter Meulen who unfortunately is not able to be here today. He conveys his best regards and wishes for an interesting and successful meeting.

The history of the German Academy of Sciences Leopoldina goes back to January 1, 1652 when it was founded by four medical doctors and was named *Academia Naturae Curiosorum*.

Its chief mission is the promotion of science, the interdisciplinary discussion of scientific issues and their presentation in the context of a biannual assembly of its members, a biannual conference as well as numerous meetings and symposia, such as yours. During the last years the Academy has developed interactions with numerous academies world-wide, most importantly the Royal Society in London, the *Académie des Sciences* in Paris and more recently the National Academies of China, India and the USA.

The German Academy of Sciences Leopoldina has a maximum of 1,000 members under the age of 75. About 70 % come from German Speaking countries, Germany, Austria and Switzerland; about 30 % from countries all over the world. The Academy is organized in 28 sections, one of which – section 13 – is your section "Microbiology and Immunology", headed by Professor Hacker whom you all know.

Many famous microbiologists are or were members of the Academy:

- For example Jacob Henle (1809–1885, L[1] 1858), who discovered that living organisms are the cause of infectious diseases. He was the teacher of Robert Koch and co-authored the Koch-Henle postulates for proving the infectious nature of an agent.
- Ferdinand Julius Cohn (1828–1898, L 1849), founder of the experimental scientific bacteriology with among others the introduction of sterilized growth media.
- Albert Neisser (1855–1916, L 1886), who discovered gonococci as cause of gonorrhea.
- Anton Weichselbaum (1845–1920, L 1888), who discovered meningococci as cause of meningococcal meningitis.

1 L = Member of the Leopoldina since.

– Kiyoshi SHIGA (1870–1957, L 1927), who discovered Shigella dysenteriae.
– Erich HOFFMANN (1868–1959, L 1933), who discovered *Treponema pallidum* as cause of syphilis.

Also, several basic scientists in the field of microbiology and cell biology were members of the Academy and received the Nobel Prize.

– Max DELBRÜCK (1906–1981, L 1963), who opened the field of bacteria genetics, Nobel Prize 1969.
– Jacques MONOD (1910–1976, L 1964), who described the operon model of gene expression, Nobel Prize 1965.
– Arthur KORNBERG (*1918, L 1964) who discovered the DNA polymerase and characterized DNA replication and repair, Nobel Prize 1959.
– André Michel LWOFF (1902–1994, L 1970), who defined the details of the lysogeny of bacteria, Nobel Prize 1965.

In recent years, the Academy, largely promoted through its President Professor Volker TER MEULEN, has shown a strong interest in infectious diseases and has issued or greatly contributed to several written statements on infectious diseases, such as:

– In 2005 a *Recommendation to Combat Infectious Diseases* and a Report of the European Academies Science Advisory Council (EASAC) on *Infectious diseases – importance of coordinated activity in Europe*.
– In 2006 an EASAC report on *Vaccines: innovation and human health* and a Joint Academy statement on *Avian influenza and infectious diseases*.
– And in 2007 an EASAC report on *Tackling antibacterial resistance in Europe* as well as the active participation in an InterAcademies Medical Panel (IAMP) Working Group in Shanghai on *Combating the threat of new emerging infections*.

Demonstrating its long-standing and strong interest in infectious diseases the Academy is happy to co-organize and co-sponsor your symposium on the occasion of the 150th birthday of Theodor ESCHERICH.

Apart from scientific advances you will have the opportunity to get to know the natural beauty and some historic places of the region, including the birth place of Theodor ESCHERICH.

On behalf of Professor TER MEULEN and the Presidium of the Academy I wish you a successful meeting, with a lively exchange of new information related to the molecular biology of *E. coli*, the pathogenesis of *E. coli* associated diseases as well as clinical aspects.

I wish you a splendid symposium and a time to remember. Thank you for your attention.

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Nova Acta Leopoldina NF *98*, Nr. 359, 13–15 (2008)

Welcome Address of the Julius-Maximilians University of Würzburg

Axel Haase (Würzburg)

President of the University of Würzburg

Dear colleagues,
Ladies and gentlemen,

On behalf of the Julius-Maximilians University of Würzburg, I should like to extend a warm welcome to all of you. It is a great honor for us that experts on *Escherichia coli* not only from many different neighboring countries of Europe and the United Kingdom, but also from the United States, Canada, and even from the Near and the Far East are attending the EMBO-FEMS-Leopoldina Symposium.

Ladies and gentlemen, we are proud to have you here. And we are also proud that the man who gave the name to the microbe – and thus to the symposium as well – is among those famous scientists who had studied and worked at our university respectively our university hospital. Theodor Escherich did part of his studies at the Julius-Maximilians University, and, after qualifying in Munich in 1881, he came back to Würzburg to become the first assistant to the internist Karl Christian Adolf Jakob Gerhardt, in our university clinic, the Julius-Spital, and there he made some of his first discoveries more than a century ago.

The 150th birthday of Theodor Escherich thus is a most welcome occasion for gathering a great number of internationally recognized authorities in this field, as well as many young scientists, who are engaged in the research on *Escherichia coli*. We all know how important this direct communication is in modern science and research – especially in the fields of medicine and molecular biology, areas where scientific discoveries are expected to lead to new ways of medical treatment and thus to improve the situation of ill persons. And this is even more the case, when the issue is of such a great interest for the researchers as *Escherichia coli* – a microbe which causes various grave infections, but which is used as a "working horse" in many scientific laboratories as well. The research on this microbe thus covers a very wide range of topics to be discussed during your symposium – including "Genomics and Physiology" as well as "Commensalism and Pathogenesis", "Virulence Factors" as well as "Host-Pathogen Interactions".

The preparation of an important meeting like this symposium demands a lot of planning and preliminary work, and I would therefore like to express my very hearty thanks to the colleagues and members of the local and international organizing committee. One look at your time-table shows that a busy week with a very interesting scientific program is ahead

of you, but it also shows that those who have organized it, made sure that you will be rewarded in the end: on Friday, excursions are planned to the small town of Ansbach, where ESCHERICH was born, as well as to Würzburg, and especially to our university hospital, the Julius-Spital, where he worked.

Both, the Julius-Maximilians University and the hospital, have part of their name in common, because both were founded by the same man, prince bishop Julius ECHTER OF MESPELBRUNN: the Julius-Spital in 1579, and the university three years later in 1582 – the university, it should be added, for the second time then, its first foundation goes back to 1402 … Both have thus a long tradition in common, and both have produced numerous well-known scientists and researchers, not least because medicine always played a very important role at our university: Already since the invitation to the medical chair of Prof. Carl Caspar SIEBOLD in 1769 the faculty of medicine had been continuously increased and reinforced by outstanding researchers and doctors. And in the second half of the 19th century – that is at the time of Theodor ESCHERICH's stay here – the "Würzburg School" of Medicine and Science was famous throughout the nation and abroad. Among its representatives, the faculty of Medicine counted as members the leading physicians of the time, especially in the physiological and anatomical field, as for example DÖLLINGER, SCHOENLEIN, KOELLIKER, VIRCHOW and RINDFLEISCH, to mention only the most important. At that time, almost half of all students of our university studied medicine. Together with medicine natural sciences, too, prospered enormously at our *Alma mater* and attracted crowds of students because of men like the zoologist SEMPER, the botanist SACHS, the chemist WISLICENUS, or the biologist Theodor BOVERI, and later on the chemists and Nobel-Prize winners Emil FISCHER and Eduard BUCHNER. And, in this context, I should also mention another outstanding son of our *Alma mater*, who was not a physician but a physicist, but whose research has proved to be very important for medicine, too. I'm talking of Wilhelm Conrad RÖNTGEN, of course, who got the first Nobel Prize in 1901 (and was thus the first of meanwhile 13 Nobel-prize winners who, during the last century, have been working at our university).

During the last decades, Würzburg has again given special regard to the activities in the medical and scientific field, so that today we are among the top research universities in these fields in Germany, providing our professors as well as our students with excellent working conditions. I might mention here: the interdisciplinary Center of Clinical Research (established in 1966), the Center for Experimental and Molecular Medicine (founded in 2003), and the Center for Operative Medicine (which followed in 2004). By 2009 a new Center for Internal Medicine will be completed too.

Besides, there are several biomedical centers, so that meanwhile a real cluster for medicine and bio-sciences has evolved at our university, ranging from molecular biology to clinical application and encompassing basic research in physics and chemistry as well as highly modern technologies.

Among our latest achievements there is the Biocentre, which was especially designed for interdisciplinary research and under which roof since 1993 scientists from three different faculties have been cooperating fruitfully. There is also the "Rudolf-Virchow-Centre for Experimental Biomedicine", a new centre of excellence that was started in 2002. It has excellent equipment, a modern infrastructure, a high amount of current funding, and, above all, excellent specialists. And there is, last but not least, of course, our Research Center for Infectious Diseases, which has been the local organizer of your symposium. It was founded in 1991 and unites research interests and activities in the field of microbiology and infectious diseases from four faculties.

Interdisciplinary research – as it is practized in these different centers – is of prime importance nowadays, because it is an essential prerequisite for the continuous advancement of science, which we so urgently need, especially in the field of infectious diseases, which continue to be a major problem throughout the world. Some years ago, a commission reporting to the World Health Organization performed a penetrating study on what the most urgent health needs of the developing countries were, and reached the conclusion that infectious diseases – in addition to nutritional deficiencies – are still the most important.

Therefore, ladies and gentlemen, once again thank you very much for coming and presenting your latest findings in the important field of *Escherichia coli*. We are glad to be your hosts, and I wish you a scientifically satisfying as well as personally enjoyable stay here in Bad Staffelstein, in Würzburg and in the surrounding country of Franconia. I hope that, when you leave at the end of the symposium, you will do so with the firm intention of coming back as soon as possible.

Prof. Dr. Axel Haase
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Nova Acta Leopoldina NF *98*, Nr. 359, 17–18 (2008)

Welcome Address of the German Society for Hygiene and Microbiology and of the Medical Faculty of the University of Würzburg

Matthias Frosch (Würzburg)

Dean of the Medical Faculty of the University of Würzburg

Magnifizenz Professor Haase,
Dear Jörg Hacker,
Dear colleagues,

It is a pleasure and distinguished honor for me to welcome you to the conference on *Escherichia coli*, which is devoted to the 150th anniversary of Theodor Escherich's birthday. I am pleased to give this welcome address on behalf of both, the German Society for Hygiene and Microbiology and the Medical Faculty of the University of Würzburg.

The German Society for Hygiene and Microbiology is the oldest society for microbiology in Germany and one of the oldest in that field worldwide. The society was founded more than 100 years ago at a time, when microbiology experienced the first heyday and at the end of the 19th century became a leading discipline in biomedical research. It was the time when bacterial species were recognized as the causative agents of contagious diseases and the great plagues threatening mankind, like tuberculosis, cholera, diphtheria and many others. The fact I found highly remarkable and impressive, was that already in these very early days of infectious disease research, the first concepts on the physiological role of bacteria were developed, and it was Theodor Escherich, who described the complex composition of the gut flora and who came up with the idea that intestinal bacteria play a pivotal role in the gut physiology and digestion. It is that themes about symbiosis, mutualism, commensalisms on the one hand and pathogenicity on the other, that determines and drives to a large extent microbiological research even today. And *Escherichia coli* has become a paradigm and a perfect model organism to study and to understand commensalisms and the evolution of bacterial pathogenicity.

For a couple of years Theodor Escherich lived in Würzburg and worked here in the Juliusspital. The Juliusspital was founded in 1576 as charity institution and hospital for poor, hungry and homeless people by Prince-Bishop Julius Echter von Mespelbrunn. This hospital also became the worldwide first university hospital as the physicians of the hospital were members of the Medical Faculty and the medical students were trained and taught here at the bedside – a completely new concept in medical education at that time. And the Juliusspital remained the University Hospital until the 1920s. Another important historical episode of the Juliusspital was, when – again worldwide for the first time – a Department for Paediatrics was founded in the Juliusspital in the 1840s. In this at that time highly innovative in-

stitution Theodor ESCHERICH received his first training in paediatrics and here his interest on the intestinal bacterial flora in infants emerged.

Obviously, there are strong links between the Medical Faculty of the University of Würzburg and Theodor ESCHERICH and his major research interest; the *Bacterium coli commune* detected and described by ESCHERICH is still a major research topic in Würzburg today. It were Werner GOEBEL, Jörg HACKER, also Helge KARCH at his Würzburg time, who devoted their scientific interests and efforts to this bacterium and who made major contributions to our understanding about *E. coli* as a commensal and as a pathogen. Therefore it is only a consequence to celebrate the 150th anniversary of Theodor ESCHERICH's birthday in the area where he originated and received his education and training as physician, and it is also a consequence that the Würzburg *E. coli* scientists brought together the internationally leading scientists working on this bacterium for a conference on the occasion of this anniversary.

Dear Jörg, I thank you for all these efforts in putting together such a great scientific program. In particular I would like to thank the local organizing committee from your group, especially Gabriele BLUM-OEHLER, Claudia BORDE and Hilde MERKERT, for the enormous work in organizing this conference and highly promising social events of the next days. All participants I wish great success, brilliant talks, stimulating discussions, warm friendship, and a pleasant time in this special environment of Kloster Banz.

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Nova Acta Leopoldina NF *98*, Nr. 359, 19–20 (2008)

Welcome Address of the "Research Center for Infectious Diseases" of the University of Würzburg

Jörg Hacker ML (Würzburg)

Speaker of the "Research Center for Infectious Diseases" of the University of Würzburg

Magnifizenz, Spektabilis,
Dear Presidents,
Ladies and Gentlemen,

As the speaker of the "Research Center for Infectious Diseases" of the University of Würzburg, I would like to welcome you to our meeting „*Escherichia coli* – Facets of a Versatile Pathogen" here at Kloster Banz.

We were listening to the excellent piano music offered by Werner Goebel ML. He selected a master piece of Franz Liszt who lived and worked in Weimar (Thuringia) not far from Kloster Banz. The landscape in Thuringia and Franconia always stimulated composers, artists, scientists and writers to create master pieces in arts, music, but also literature and science. Thus, Johann Wolfgang von Goethe lived in this area and he described in a dialogue with Eckermann the development of research in his age with the following sentence: "Modern Research cannot be done alone, you need a group of people with similar ideas and spirit."

This is exactly, what we are doing here at Kloster Banz with our meeting on *Escherichia coli*. We bring together basic scientists, scientists working in the field of medical microbiology as well as clinicians. Furthermore, there are different institutions involved in the organization and support of our symposium. First, I would like to thank EMBO, the European Molecular Biology Organization, for the support of this meeting. Furthermore, the Federation of European Microbiological Societies (FEMS) is acting as one of the organizers, and we are very proud that the president of FEMS, Eliora Ron (Tel Aviv/Israel), is here with us. Furthermore, the University of Würzburg is represented by the president, Prof. Haase, and the Dean of the Medical Faculty, Prof. Frosch. The University of Würzburg with its "Research Center for Infectious Diseases" is very instrumental regarding the organization of the meeting. Furthermore, the German Academy of Sciences Leopoldina, which is represented by the board member, Prof. Blum ML (Freiburg), was actively involved in the organization of the meeting. The microbiological societies of Germany, especially the *Deutsche Gesellschaft für Hygiene und Mikrobiologie* (DGHM) and the *Vereinigung für Allgemeine und Angewandte Mikrobiologie* (VAAM) are represented by the former president, Prof. Frosch, and the president of the DGHM, Prof. Heesemann ML. Furthermore, a number of companies support our meeting and we are very grateful for their help. In addition, I would

like to welcome all the speakers and participants, and I would especially like to welcome Julian DAVIES and Agnes ULLMANN, who together with James KAPER will give the welcome speeches. Julian DAVIES from Vancouver, a worldwide-known scientist, is the former president of the American Society for Microbiology (ASM) and the International Union of Microbiological Societies (IUMS). Agnes ULLMANN, who contributed to the molecular biology of *Escherichia coli*, is the former scientific director of the Institut Pasteur, Paris.

The idea to organize the meeting was born on the basis of the fact that the discoverer of *Escherichia coli*, Theodor ESCHERICH, was born in Ansbach, which is not far from Kloster Banz, 150 years ago. He initiated research on *Escherichia coli*. Furthermore, our EU-program "COLIRISK", which was running from 2002 to 2006, represents an umbrella for the meeting as well as the EU-Network of Excellence "EuroPathoGenomics". The current knowledge of *Escherichia coli* will be summarized by 31 lectures and 69 poster presentations. More than 180 participants from 22 countries including scientists from a number of non-European countries will discuss the current knowledge and future developments in the world of *Escherichia coli.*

I would like to thank especially Gabriele BLUM-OEHLER, Claudia BORDE and Hilde MERKERT, who are in charge of the organization of the meeting. Furthermore, I would like to thank the staff of the "Bildungszentrum Kloster Banz", which represents an exceptional place to stay. As a former monastery, it was developed into a conventional center run by the "Hanns-Seidel-Foundation". I guess, the spirit of Kloster Banz will support the exchange of knowledge and ideas. Therefore, I wish all of you a nice time, a successful meeting and fruitful discussions at Kloster Banz.

Thank you for your attention.

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Welcome Address of the Federation of European Microbiological Societies

Eliora Z. Ron (Tel Aviv, Israel)

President of the Federation of European Microbiological Societies

Dear Professor Hacker,
Professor Blum,
Professor Haase,
Professor Frosch,
Ladies and gentlemen,

I wish to thank the organizers of this meeting for an excellent program and an outstanding administration. I would also like to extend my thanks for the support from the Leopoldina Academy and EMBO.

It is an honor to represent FEMS in this exciting birthday party. FEMS is the Federation of European Microbiological Societies and links 47 microbiology societies in 36 European countries with approximately 30 000 Microbiologists. The main activities of FEMS are publications – the society published 5 journals – and support of microbiologists, with special emphasis on the support of young scientists.

I am glad to tell you that in its last Council meeting FEMS approved two important initiatives

- European Microbiology Council – to strengthen the impact of microbiology in the public opinion and European Community institutes.
- The establishment of a European Academy of Microbiology which will be a leadership group of European microbiologists that will increase the academic impact of microbiology in Europe.

I believe that these two new initiatives will improve our visibility and significance both academically and politically.

I wish us all an interesting and productive meeting.

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Theodor Escherich and *Escherichia coli*

Nova Acta Leopoldina NF *98*, Nr. 359, 25–28 (2008)

Theodor Escherich, the Bacteriologist

Tobias A. Oelschlaeger (Würzburg)

Abstract

Theodor Escherich (1857–1911) was born in Ansbach and studied medicine at the University of Würzburg. He became medical assistant at the Julius Spital in Würzburg in 1883. Here he became interested in pediatrics and started his first bacteriological studies (1884) at the pathology in Vienna during a three month study visit. Back in Bavaria, Escherich continued his studies on the microorganisms of stool of infants. He summarized the results in his postdoctoral thesis (Habilitation), published 1886, where he reported in detail the properties also of "*Bacterium coli commune*". In 1894 he demonstrated *E. coli* to be a causative agent for cystitis.

Escherich not only identified *E. coli* but also was the first to describe *Campylobacter* in a publication (1884) after his study visit in Naples aimed to monitor the cholera epidemic. Unfortunately, Escherich never fully realized the importance of *Campylobacter* as a pathogen.

Zusammenfassung

Theodor Escherich (1857–1911) wurde in Ansbach geboren und studierte an der Universität Würzburg Medizin. Er wurde 1883 medizinischer Assistent am Julius-Spital. Dort wurde sein Interesse an der Pädiatrie geweckt, und er begann während eines dreimonatigen Studienaufenthalts in der Pathologie in Wien seine ersten bakteriologischen Studien (1884). Zurück in Bayern setzte Escherich seine Arbeiten mit Mikroorganismen im Stuhl von Kleinkindern fort. Er fasste die Ergebnisse dieser Studien in seiner Habilitationsschrift 1886 zusammen. Darin berichtete er auch detailliert über die Eigenschaften von „*Bacterium coli commune*". Im Jahre 1894 gelang es ihm, *E. coli* als einen Verursacher von Cystitis zu identifizieren.

Escherich identifizierte nicht nur *E. coli*, sondern war auch der erste, der im Nachgang zu einer Reise nach Neapel, die eigentlich dem Monitoring einer Choleraepidemie diente, *Campylobacter* beschrieb (1884). Unglücklicherweise erkannte Escherich die Bedeutung von *Campylobacter* als Krankheitserreger nicht.

Theodor Escherich was born in Ansbach in 1857 as the first child of "Medizinalrat" Ferdinand Escherich and his third wife Maria. Five years later in 1862 Theodors mother died shortly after the birth of her second son Ferdinand. Another five years later the family moved to Würzburg. There Theodor's father got the position as a "Kreismedizinalrat". In summer 1876 Theodor finished the humanistic high school in Würzburg with the general qualification for university entrance ("Abitur"). After a 6 month military service in Strassburg, Escherich started his academic and medical education at the University of Würzburg in winter semester 1876. At the beginning of his 5th semester he passed the predegree examination and went for one semester to Kiel and later on to Berlin. The three last

semesters he was back in Würzburg where he passed the final exam and got the license to practice medicine at 16th December 1881. After finishing the second part of his military service as physician at the garrison hospital in München-Oberwiesenfeld he was appointed second assistant of Karl Gerhard, head of the medical department at the Julius Spital in Würzburg. Gerhard as cofounder of pediatrics initiated Escherich's interest in this new discipline. In 1883 he graduated with Gerhard as his supervisor and became a first assistant. However, already in Mai 1883 Escherich quitted this position in order to specialize in pediatrics. For that purpose he traveled to Vienna to attend the lectures of the pediatrician Hermann von Widerhofer. Widerhofer was the chief of the *St. Anna-Kinderspital*, the oldest children hospital in Vienna. Most likely at that time Escherich finally decided to become a pediatrician. Escherich introduced himself to Heinrich von Bamberger with a letter of recommendation from Gerhard. In the Institute of Pathology, headed by Bamberger, Escherich started his bacteriological work in Vienna. Among others he performed bacteriological analysis of breast milk samples. He discovered that breast milk is sterile except if the mother is febrile. In the milk of febrile mothers he found "certain cocci". Escherich even succeeded to identify yellow and white colonies of such cocci: these were most likely *Staphylococcus aureus* and *S. epidermidis* colonies. Astonishingly, he did not elaborate on effects on the puerperal process.

After a short trip to Naples (1884) in order to study a cholera outbreak Escherich moved from Würzburg to Munich. He had chosen Munich, because there pediatrics had been accepted as a separate discipline. At the University of Munich Escherich continued his bacteriological studies. Of great advantage was the presence of Wilhelm Frobenius at the Munich Institute of Pathology, because Frobenius was a student of the famous bacteriologist Koch and taught Escherich the technique of pure culture and how to characterize bacteria. In a talk in 1885 he presented first results of his bacteriological studies on the impact of gut bacteria on physiology and pathology. In December of the same year Escherich developed a new technique for bacteriological analysis of juvenile stool. Using this he was able to isolate "10 bacilli species, 5 cocci, several sarcina and yeast." He also found meconium to be sterile, and the first bacteria in the newborns gut he detected after 4 to 7 h post partum. In 1886 Escherich published the results of his research regarding the microflora of the juvenile gut indicating that he has started this work already in Würzburg. In this publication he described in detail 19 different bacterial species regarding their morphology and cultivation conditions. Specifically he elaborated on what he termed *Bacterium coli commune*. He had found this species in almost pure culture in the faeces of breast fed infants. This was due to the lack of cultivating obligate anaerobes although he showed this species to be able to replicate under anaerobic conditions. Later on Escherich was able to detect *Bacterium coli commune* also in the faeces of adults. Castellani and Chalmers proposed already in 1919 to rename this species in honor of T. Escherich as *Escherichia coli*. This was finally officially acknowledged in 1958.

However, Escherich was also the first to describe what we know nowadays as the genus *Campylobacter*. This work started during his trip to Naples in 1884. He mentioned already in a talk that year this helical bacterial genus as part of the stool flora from patients which had come down with cholera. The term he used for these bacteria was "Zahnspirochäten". Later in the same year he even identified *Campylobacter* in the deeper mucus layers of the colon in 16 out of 17 children which had died of gastrointestinal diseases. However, he believed *Campylobacter* not to be involved in the etiology of the observed intestinal diseases.

Instead he interpreted their presence in the gut of the dead children as an overgrowth phenomenon resulting from a pathological alteration of the gut.

ESCHERICH's research and the resulting publications made him well known, and therefore, he was offered already at the age of 33 the position of professor extraordinary of pediatrics at the *St. Anna-Kinderklinik* in Graz, Austria. From 1890 to 1902 he transformed this unknown provincial hospital into a modern, internationally recognized one by greatly improving the hygiene standard, acquiring an X-ray machine for diagnosis in 1897 (the x-rays were discovered by Wilhelm Conrad ROENTGEN 1895 in Würzburg) and by employing "Serum Therapy" to treat diphtheria. The high hygiene standard was most prominent in the 1899 founded baby ward. There, healthy infants were separated from the diseased ones. The commodities of each child were placed in its own small box above the bed. These measures aimed to prevent infections were rather modern at this time. It should not be concealed that ESCHERICH was not only an excellent bacteriologist and pediatrician but also a talented and successful organizer. This was the prerequisite to get enough funding for the updating of the *St. Anna-Kinderklinik*.

Of special interest in the context of the symposium is ESCHERICH's report about what he called colicystitis. He reported in 1894 about seven girls with cystitis and the isolation of *Bacterium coli commune* from the urine of these girls. He concluded that *Bacterium coli commune* was the causative agent of cystitis. He supported this conclusion by the observation, that these bacteria were only detectable as long as there were symptoms. This clearly shows that ESCHERICH was already aware, that besides commensal *E. coli* strains there are also pathogenic strains.

After the death of WIDERHOFER, ESCHERICH was offered the chair in pediatrics at the University of Vienna. The *St. Anna-Kinderspital* of Vienna was the third oldest pediatric clinic founded in 1837. ESCHERICH accepted this offer in 1902 only after the officials in Vienna had agreed to renovate the old building and to plan construction of a new hospital. He himself made detailed plans for a modern new building with quarantine wards, an x-ray diagnostic unit and a chemical and – most importantly – a bacteriological laboratory.

ESCHERICH spend also a lot of effort and time to bring the problem of the high infant mortality into public awareness. His clever and charming persuasiveness was the basis for many projects aimed on preventive measures. For that purpose he founded in 1904 the association for infant protection ("Verein Säuglingsschutz"). This association supported efforts to promote breast feeding, educate mothers of the underclass and establish a distribution system for milk. Furthermore, this association enabled ESCHERICH to open the first infant ward of all seven pediatric hospitals in Vienna. Usually infants were not hospitalized due to the high mortality rate (> 80 %!) and the fact that only for children from the age of 4 the hospitals received financial support. In his infant ward the principles cleanliness and asepsis resulted in a dramatic reduction of the death rate. Of equal importance were for ESCHERICH well educated nurses. Therefore he also founded a training school for nurses associated with the infant ward.

At February 15th in 1911, his new hospital was just the shell of building, ESCHERICH died at the age of 54 of an apoplectic shock. He was buried two days later at the Hernalser cemetery in Vienna. His death left behind a serious gap in the field of bacteriology as well as in the line of those who were fighting for the health of juveniles and their mothers.

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Theodor Escherich and the Emergence of Molecular Biology

Agnes Ullmann (Paris)

With 1 Figure

Abstract

Discovered in 1885, *Escherichia coli* played an important role in the emergence and development of modern molecular biology. It originated in the creation of the "Phage Group" in 1940 by A. D. Hershey, S. E. Luria, and M. Delbrück at Cold Spring Harbor. *E. coli* and its viruses became the basis of genetic studies, leading between 1943 and 1946 to seminal discoveries, such as the spontaneous nature of bacterial mutations by Luria and Delbrück, bacteriophage recombination by Delbrück and Hershey and bacterial sexuality by Lederberg and Tatum. Molecular biology in Europe started to develop after World War II: by the end of the 1950s the mechanism of the genetic control of enzyme and virus synthesis was elucidated by Lwoff, Monod and Jacob.

Zusammenfassung

Escherichia coli, ein Bakterium das 1885 entdeckt wurde, spielte eine wichtige Rolle in der Entstehung und der Entwicklung der modernen Molekularbiologie. Die Anfänge gehen auf die 1940 von M. Delbrück, S. E. Luria und A. D. Hershey in Cold Spring Harbor gegründete „Phage Group" zurück. Damals wurden *E. coli* und seine Viren zur Basis genetischer Studien, die dann zwischen 1943 und 1946 zu bahnbrechenden Entdeckungen führten: Luria and Delbrück erkannten die spontane Natur bakterieller Mutationen, Delbrück und Hershey entdeckten die Bakteriophagenrekombination und Lederberg und Tatum die bakterielle Sexualität. In Europa entwickelte sich die Molekularbiologie nach dem Zweiten Weltkrieg: Ende der 1950er Jahre wurden der Mechanismus und die genetische Kontrolle der Enzym- und Virussynthese von Lwoff, Monod und Jacob aufgeklärt.

Theodor Escherich, at the age of 28, published two papers on the colonization of the intestines of neonates by bacteria. He isolated and obtained practically pure cultures of "slim, occasionally slightly curved, short rods" that were Gram-negative, slightly motile and grew well on many of the artificial media available at the time (Escherich 1885a,b). Escherich named these organisms *Bacterium coli commune*, known since 1919 as *Escherichia coli*. In 1907, Rudolf Massini described an organism, named *Bacterium coli mutabile*, that he obtained from a strain which, lactose-negative on primary isolation, developed papillae that did ferment lactose (Massini 1907). This finding was probably the basis of many subsequent studies on the molecular biology of *E. coli*.

The development of what we call nowadays molecular biology originated in the creation of the "Phage group" in 1940 by Max Delbrück, Salvador E. Luria and Alfred D. Hershey at Cold Spring Harbor. At the same time, *Escherichia coli* and its viruses became the materials of choice for basic studies in molecular genetics.

Delbrück, a German quantum physicist, arrived in the United States as a Rockefeller fellow and went to Caltech to work on phage genetics with Emory L. Ellis. In 1939 they published the first quantitative description of phage multiplication, known as the one-step growth experiment (Ellis and Delbrück 1939).

Luria, an Italian medical doctor, left fascist Italy in 1938 for Paris. In 1940 he had to leave France, and he succeeded to arrive in the USA. Soon after his arrival he met Delbrück, and they decided to collaborate on the nature of phage-resistance in bacteria to answer the question: Did resistance result from mutation followed by selection or from adaptation induced by exposure to the phage? The experiment of Luria, known as "fluctuation test", consisted in the inoculation of a small number of bacteria (*E. coli* B) into separate culture tubes. After a period of growth, equal volumes of these separate cultures were plated onto plates saturated with T1 phage. If phage resistance began only at the moment of exposure of the bacteria to the phage, then all plates should show the same distribution of resistant clones. The experiment displayed a large fluctuation from the average count, providing evidence that phage-resistant mutants originated by spontaneous mutation and not as a result of phage attack. The paper of Luria and Delbrück (1943) certainly marks the beginning of modern bacterial genetics.

Alfred Hershey joined the "Phage group" in 1943. He was already known for his work on genetics with T-even phages of *E. coli*, but his name is linked to the famous *Hershey-Chase Experiment* (1952) done with Martha Chase, which demonstrated the role of DNA in phage infection.

The group of phage workers at Cold Spring Harbor, under the influence of Delbrück, decided to concentrate research on a set of T-phages, active against the same host, *Escherichia coli* strain B. This agreement was the so-called "phage treaty of 1944". One of these phages, T4, was brilliantly exploited by Seymour Benzer in his classical studies on the rII system, that let him define the gene functionally with the "*cis-trans*" test showing that the unit of function is the "cistron", which we now equate to the gene (Benzer 1955). It is of interest to note, that many years later, using the same rII system, Brenner and Crick by combining deletion, addition and frameshift mutations, were able to generate a functional rII gene when groups of 3 plus or 3 minus mutations were brought together. Therefore, before the actual code was known, they could conclude that the translating frame read the code by triplets (Crick et al. 1961). T4 mutants were also instrumental in demonstrating the co-linearity of the gene with the polypeptide chain (Sarabhai et al. 1964).

In the summer of 1945, Delbrück started the first "phage course" that went on for 26 years. Most of those who, in the first years, took this course at Cold Spring Harbor (Rollin Hotchkiss, Hermann Kalckar, Leo Szilard, Aaron Novick, Seymour Benzer, Gunther Stent, Giuseppe Bertani, Norton Zinder, Wacław Szybalski, Frank Stahl, Bob Edgar) later went on to make the most significant contributions on phage or other aspects of molecular biology. In 1969, Delbrück, Luria and Hershey were awarded the Nobel Prize for Physiology or Medicine.

The famous Cold Spring Harbor Symposia on Quantitative Biology were published for the first time in 1933. At the 1946 symposium, Delbrück and Hershey revealed their discoveries on phage recombination, at the same time that Lederberg and Tatum announced their discovery of bacterial sexuality, introducing a new *Escherichia coli* strain, K-12, which was used for almost all subsequent research in bacterial genetics.

Edward Tatum was known from his work with George Beadle on metabolic mutants of *Neurospora crassa.* They demonstrated that genes determine the structure of specific en-

zymes and proposed the "one gene – one enzyme" hypothesis (BEADLE and TATUM 1941). From 1945 on, TATUM went on to study genetics in *Escherichia coli*. In 1946, with his Ph.D. student, Joshua LEDERBERG, 20 years at that time, using nutritionally deficient double mutants and selecting for prototrophic recovery, they showed that bacteria could share genetic information through bacterial conjugation (LEDERBERG and TATUM 1946). In 1958 LEDERBERG, BEADLE and TATUM got the Nobel Prize for Physiology or Medicine.

At the same 1946 Cold Spring Harbor Symposium, André LWOFF, representing the after-war Pasteur group, was also present. During World War II, LWOFF's laboratory was an active center of the Underground. Jacques MONOD spent some time there clandestinely to perform a few experiments on "enzymatic adaptation". In his lecture, LWOFF gave an account of some experimental results obtained during the war period. Among others, he related MONOD's first remarkable discovery, made under the German occupation: the phenomenon of diauxy (MONOD 1941). Growing bacteria on two carbon sources, instead of a single one, in some mixtures of two sugars when one of them was glucose, MONOD observed two distinct growth cycles, separated by a lag phase. He realized that the diauxic growth was the result of the inhibitory action of glucose on the formation of the then called "adaptive enzymes" that metabolize the second carbohydrate (today we call this catabolite repression), and the lag phase corresponded to the time necessary for the induction of these enzymes.

MONOD joined in 1943 the armed resistance movement, became chief of the National Headquarters, and after the liberation of Paris, in 1944, he joined the free French forces.

The war ended and MONOD joined the "Service de Physiologie Microbienne", the famous attic, where LWOFF had brought together a remarkable staff which included besides MONOD, Elie WOLLMAN and later François JACOB. A great number of foreign scientists, including Melvin COHN, Annamaria TORRIANI, Martin POLLOCK, Alvin PAPPENHEIMER, Mike DOUDOROFF, Dale KAISER, Dave HOGNESS, Seymour BENZER and many others joined LWOFF's laboratory. The "Golden Age" of molecular biology started at the Pasteur Institute.

MONOD started to work on the β-galactosidase of *E. coli*, isolated a number of *lac* mutants, and in a few years, by using gratuitous inducers (lactose analogs), he uncovered the mechanism of β-galactosidase induction. Studying the relationship between gene and enzyme, MONOD and collaborators established that in *E. coli*, the synthesis of β-galactosidase depends on two genes: *z*, governing the capacity or incapacity to produce the enzyme, *y*, governing the synthesis of lactose permease, and on a genetic factor, known to exist under the forms i^+, wild type, corresponding to inducibility, and i^-, corresponding to constitutivity. Genetic analysis revealed that the *z*, *y* and *i* genes are closely linked (MONOD and COHN 1952).

Around the same period, André LWOFF elucidated the mechanism of lysogeny: when a bacteriophage infects a bacterium it can enter either lytic or lysogenic cycle. In the lysogenic bacterium the phage DNA becomes integrated into the bacterial chromosome, as a prophage, as LWOFF named it, and replicates along with it. Induction of the prophage (UV radiation) would lead to multiplication and release of the phage (LWOFF 1953).

François JACOB joined LWOFF's laboratory in 1950. At the beginning of the war he was a medical student and wanted to become a surgeon. In 1940 he joined the free French forces in London, and after having participated in the Africa campaign with the Division LECLERC, he was severely injured during the Normandy landing in 1944. Because of his injury he had

to give up the idea of becoming a surgeon. But he had the chance to start research, as he recalls, at the good place just at the right time. He arrived just after the discovery by LWOFF of the induction of phage production. He started his research by a genetic analysis of lysogeny. With WOLLMAN, taking advantage of the discovery by CAVALLI and HAYES of Hfr mutants that transfer the genetic material to female bacteria (F^-) with high frequency, they elucidated the mechanism of the sexual process, demonstrated the circularity of *E. coli* chromosome, thus providing new and powerful tools to attack the problem of regulation (JACOB and WOLLMAN 1956).

In 1957 started an extremely fruitful collaboration between MONOD and JACOB. They decided to use a genetic approach, i. e. conjugation for a genetic analysis of the lactose system. The *i*, *z* and *y* mutants isolated by MONOD were then inserted in various combinations in either male or female bacteria. With Arthur PARDEE, who has spent a sabbatical year at *Pasteur*, JACOB and MONOD performed one of the most famous experiments in molecular biology, known as the PaJaMo (or PyJaMa) experiment (PARDEE et al. 1959). This experiment involved measuring the synthesis of β-galactosidase in zygotes resulting from the conjugation of male bacteria carrying the z^+ and i^+ genes with females, carrying z^- and i^- genes. The main outcome of the PaJaMo experiment was that the i^+ gene is dominant over the i^- gene, leading to the model of negative regulation: the i^+ gene produces a factor, called "repressor" that blocks the expression of the z^+ gene. The rapid expression of the z^+ gene in the i^- cytoplasm was to become the experimental basis for the development of the messenger RNA model, which stated that the information from DNA was transferred first to a metabolically unstable RNA that, in turn, will be translated into polypeptide chains on the ribosomes.

In 1961, MONOD and JACOB presented a model for the regulation of gene expression, the operon model. To account for the specificity of action of the repressor, a new structure has been proposed: the "operator". A single operator controls the expression of the adjacent *z* and *y* genes of the lactose system in a coordinate manner. Mutations inactivating the operator – unable to recognize the repressor – were isolated. The operon, a unit of coordinate transcription, was born. An operon is composed of structural genes, connected to an operator, subject to the action of a repressor, produced by a regulator gene. The binding of an inducer to the repressor will disrupt its interaction with the operator and transcription of a polycistronic mRNA will take place (JACOB and MONOD 1961). A few years later, another indispensable element, the "promoter", which was needed for expression, has been identified as the site of transcription initiation (Fig. 1).

Within ten years, the problems posed by the induced synthesis of β-galactosidase in *E. coli* had been solved. The new ideas were applied to a large number of catabolic and anabolic pathways as well as to viral development. The nature of molecular communications was clarified. LWOFF, MONOD and JACOB were awarded in 1965 the Nobel Prize in Physiology or Medicine.

The structure of DNA was discovered by WATSON and CRICK (1953), based in part on CHARGAFF's rule (G = C and A = T). WATSON and CRICK ended their paper with the famous sentence: "It has not escaped our notice that the specific pairing we have postulated immediately suggests a copying mechanism for the genetic material."

Several replication mechanisms were, in fact, possible: conservative, semi-conservative and dispersed. Two young postdoctoral fellows, Matthew MESELSON and Frank STAHL (1958), in an elegant experiment, growing *E. coli* in heavy and light nitrogen

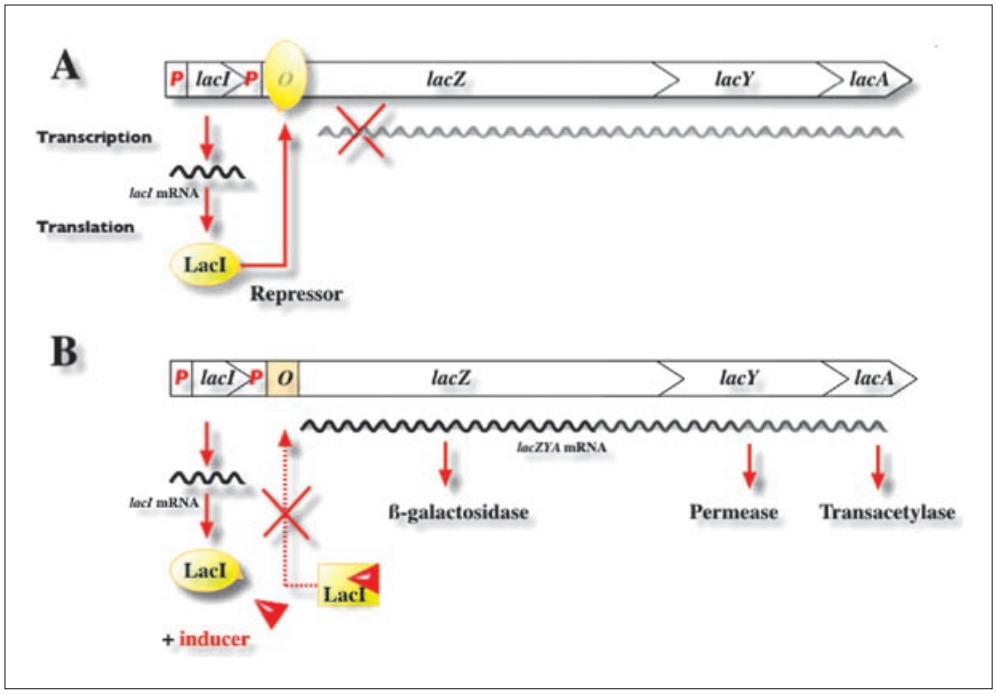

Fig. 1 The operon model. The lactose operon in the repressed (top) and induced state (bottom). In the absence of inducer, the LacI repressor binds the operator and prevents the expression of the *lac* genes, while in the presence of the inducer, the LacI repressor is inactivated and *lac* genes are expressed (drawing courtesy of Jean-Marc GHIGO)

media followed by density gradient centrifugation, proved that DNA replication was semi-conservative: when the double stranded DNA was replicated, the daughter double stranded helices consisted of one strand coming from the original helix and one newly synthesized.

Escherichia coli and its phages were the prominent actors of the development of recombinant DNA technology, based mainly on the discovery of restriction enzymes. Werner ARBER in 1965, while studying host-controlled modification of lambda, a phenomenon discovered by LURIA in the early 1950s, known also as phenotypic modification, showed that the host which restricted lambda had an enzyme that degraded DNA (restriction enzyme). This restriction enzyme did not degrade the DNA if it had been modified by methylation. Thus the restriction and modification exist as a paired system, the function of which is to protect host DNA but destroy foreign DNA (ARBER 1974). For the discovery of restriction enzymes ARBER was awarded the Nobel Prize in 1978, shared with Daniel NATHANS and Hamilton SMITH.

In 1997, Fred BLATTNER and his group published the complete genome sequence of *Escherichia coli* K-12 (BLATTNER et al. 1997); a cornerstone, that allows us to anticipate new discoveries about an organism, which for many decades played a central role in the "golden age" of molecular biology.

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Escherichia coli and the History of Antibiotic Resistance

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Abstract

Antibiotic resistance development is a complex genetic, biochemical, and environmental problem. Little is known of its origins or evolution. Would increased knowledge of these factors lead to methods of delaying resistance development in bacterial pathogens such as *E. coli*?

Zusammenfassung

Die Entwicklung der Antibiotikaresistenz ist ein komplexes genetisches, biochemisches und umweltbedingtes Problem. Über die Herkunft und Entwicklung von Antibiotikaresistenzen ist wenig bekannt. Die Frage ist, ob eine detailliertere Kenntnis dieser Faktoren zur Entwicklung von Methoden führen würde, die die Resistenzentwicklung in bakteriellen Pathogenen wie z. B. *E. coli* verlangsamen könnte.

Escherichia coli is a highly virulent pathogen and the causative agent for an increasing variety of human and animal diseases; most of these infections are now resistant to multiple antibiotics. In effect, antibiotic resistance determinants and *E. coli* have co-evolved for nearly 70 years, the period popularly known as the "antibiotic era", which could equally well be called "the era of the rise and fall of antibiotics"! Resistance to sulfa drugs, the first widely used antimicrobials, was detected in patients soon after their introduction in the late 1930s; the genetics and biochemical mechanisms of this resistance have been well studied. Next came the discovery of penicillin. Even before it was introduced into extensive clinical practice in the 1950s, Abraham and Chain reported a mechanism for resistance to penicillin with the identification of penicillinase, an enzyme produced by a strain of *Bacterium coli* (now appropriately named *Escherichia coli*) that catalyzed the hydrolysis of the β-lactam ring in the molecule and destroyed the antibiotic activity. The name penicillinase has now been replaced by the more inclusive β-lactamase, following the identification of many other bacterial enzymes that inactivate the dozens of novel penicillin and cephalosporin derivatives introduced into clinical practice in response to resistance development.

Following the discovery of penicillin, the antibiotic industry expanded rapidly. In the US in the early 1940s antibiotic production was small, but the number has risen to 25 million pounds of antimicrobials in 2005. The major producing countries are now India, China, and Russia, and actual worldwide production is difficult to estimate. It is quite possible that the quantity of antibiotics currently made by the pharmaceutical industry surpasses the nat-

ural production of antibiotics in the biosphere and has done so for many years. Most antibiotic-producing organisms initially make only small amounts of active compounds in the laboratory; the useful strains are then 'improved' for industrial production. Over the past 50 years, industrial strains have been developed that produce more than 5000 times as much antibiotic as the original soil isolate. Selection pressure for antibiotic resistance continues to be very strong in environmental, agricultural, and clinical arenas!

Some dozen different biochemical mechanisms of antibiotic resistance have been identified to date; bacteria can avoid death or inhibition by inactivating the drug (as with β-lactamases), modifying the drug target, or preventing the accumulation of the drug inside the cell. Two distinct genetic processes are known to lead to resistance: gene mutation and gene transfer/acquisition. Horizontal gene transfer, an unexpected and somewhat controversial mechanism at the time of its discovery, is a well-recognized process and is now believed to have played an important role in genome evolution. A developmental pattern has been observed with all antimicrobial use: mutation to resistance precedes gene acquisition. It appears that initially low levels resulting from mutation predispose to more clinically significant resistance, usually by gene acquisition. This stepwise process is clearly correlated with the increased use of antibiotics (Davies 2007).

The molecular evolution of resistance genes coincident with the production and use of modified recalcitrant compounds has been well charted. This is essentially an arms race, pitting the pharmaceutical industry against pathogens. The Gram-negative β-lactamases are the ultimate example of the power of natural protein engineering combining with lateral gene transfer and recombination: with each successive introduction of a new class of β-lactam antibiotic, resistance rapidly evolves. The development of the new CTX-M classes is especially illustrative; some 200 publications on this topic have been published in the past four years (Bonnet 2004)! This is a real-time evolutionary process that, coupled with efficient processes for horizontal gene transfer between bacterial genera, leads to rapid dissemination of the new resistance determinants. The result has been alarming increases in resistance to the 3rd generation cephalosporins in hospitals and communities worldwide **(**Livermore et al. 2007). The pattern has been typical for all clinically significant resistance; are genes for virulence acquired and transferred coincidentally? The presence of antibiotics in the environment and co-selective markers on plasmids is favorable to the natural genetic engineering of new bacterial hosts.

While on the one hand one cannot help but marvel at the extraordinary genetic dexterity of pathogenic bacteria such as *E.coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and their ability to survive under adverse conditions (high concentrations of antimicrobials), on the other hand it is amazing that humans should have allowed this to happen! The inexorable development and spread of resistance worldwide over the last half century has occurred with only half-hearted international action or policies instituted to prevent and control resistance development. WHO, CDC, FDA, EEC, and other agencies have long been well aware of the problem (since 1950!) and have vigorously proposed measures to curtail it, but it is evident that these well-intentioned efforts have failed to have any major effect. Uncontrolled non-therapeutic antibiotic use in animal husbandry, agriculture, and aquaculture has been practiced for some time worldwide and is still accepted in many countries, despite recommendations and/or laws to the contrary. That industrialized nations have not been able to come up with more effective ways of controlling antibiotic use in hospitals and the community is incomprehensible. This is a different but important form of drug abuse!

The origins and evolution of acquired resistance genes are not known and there has been little research on the ecological aspects of the problem. It is now well established that a vast reservoir of potential resistance genes exists in soil and marine environments; this has been called the "resistome", and a number of studies show that homologues of many of the common classes of antibiotic resistance genes are widespread in microbes in pristine environments (D'Costa et al. 2007). The increasing availability of bacterial genome sequences has revealed the ubiquity of potential resistance determinants. Antibiotic resistance may not be the normal function of these genes; they likely have other metabolic roles in their natural hosts, as has been demonstrated in several instances. Nonetheless, when cloned and over-expressed in a surrogate host, they act as typical antibiotic resistance genes. Thus, resistance determinants probably exhibit different effects depending on their concentration or level of expression. There is good reason to believe that many of the known antibiotic resistance determinants had their origins in the actinobacteria, a large phylogenetic family that includes the major antibiotic-producing bacteria, such as the high-GC-content streptomycetes, although much needs to be learned about the processes by which such genes were functionally transferred and tailored for efficient heterologous expression in their present hosts such as *E. coli* (most with genomes of lower GC content), which inhabit independent and very restricted environments. Related to this issue is the suggestion that antibiotic resistance genes and genes for bacterial virulence may have common environmental origins. Is there a causal and evolutionary relationship between resistance and virulence? One possibility is that resistance genes originated in plants; similarly, the progenitors of bacterial virulence genes may have been involved in plant pathogenesis (Pitman et al. 2005).

A variety of transmissible elements such as plasmids and bacteriophages are known to encode virulence and/or antibiotic resistance. In the case of pathogenicity, the inheritance of clusters of interacting, inter-regulated gene functions leads to the formation of multifunctional pathogenicity islands. Likewise, resistance is primarily acquired in the form of plasmids encoding one or more determinants. The pre-eminent gene-capture system, playing a key role in the development and dispersion of antibiotic resistance in the *Enterobacteriaceae* is the integron, a beautifully simple DNA combination consisting essentially of a gene that encodes an integrase that catalyzes recombinational insertion between an adjoining attachment site and the cyclic form of an antibiotic resistance gene (cassette) (Mazel 2006). Part of the integrase sequence just upstream of the attachment site encodes a strong constitutive promoter; insertion of an open reading frame (encoding resistance, virulence or other function) into the attachment site ensures expression in a wide range of bacteria! Integrons create "resistance islands" that are mobilized by elements such as plasmids or bacteriophage. In a sense they are also resistance operons, and integrons encoding up to six resistance determinants have been found. The existence of free cassettes remains hypothetical and while the integrase/attachment mechanism resembles phage lysogeny, its actual origins have not been identified. Only a small proportion of the identified gene cassettes encode resistance to a known antibiotic; the vast majority are diverse sequences with no known orthologues. It has been suggested that the gene cassettes encode functions important to bacterial adaptation and genome evolution (Boucher et al. 2007).

It is well established that resistance can be phenotypically costly to the bacterial host; genetic compensation must occur for the organism to be environmentally competitive. Not surprisingly this process remains a mystery; what are the metabolic consequences

of the acquisition of antibiotic resistance genes and mutations? How are these events integrated with the highly networked system of interactive pathways that constitutes cell metabolism (Keseler et al. 2005)? What happens when a bacterial cell inherits a resistance mutation or plasmid or is lysogenized by a bacteriophage carrying pathogenicity determinants? How do the new gene functions integrate into the existing, compatible networks? Are plasmids autonomous to the extent that they can switch hosts and cytoplasms without the need to "network"? Are plasmid functions independent, non-host-interactive networks? Acquisition by a network-incompatible host would seem to be an unstable configuration.

The most compelling reason for studying antibiotic resistance mechanisms and their genetics is to try to devise ways of overcoming the problem. Sadly, this appears to be unachievable: so long as selection is applied to bacterial populations, resistant strains will emerge and flourish; the process cannot be prevented. However, it should be possible to delay resistance development; for example, strict regulation of antibiotic usage should ensure that antibiotics have a longer useful lifetime (Finch and Hunter 2006). If policies to restrict inappropriate and unnecessary use of antibiotics can be combined with successful programmes of novel antibiotic discovery, effective antimicrobial therapy can be assured for generations to come. Nonetheless, the treatment of infectious diseases will always be an "arms race" (Nathan and Goldberg 2005).

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Natur und Migration

Vorträge anlässlich der Jahresversammlung vom 5. bis 7. Oktober 2007 zu Halle (Saale)

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„Natur und Migration" – assoziiert sehr verschiedenartige Phänomene, die sich durch Wanderungsprozesse auszeichnen. In diesem Band wurden besonders interessante Gebiete ausgewählt, u. a. Migration und Seuchen, Reisen und Epidemien in einer globalisierten Welt, der Vogelzug, aber auch die Migration geologischer Fluide, die Elektronenmigration in Halbleitern, die Migration als treibende Kraft in der Organogenese, die Biophysik der Zellbewegungen, die Migration von Tumorzellen, Migration als Phänomen in der Neurobiologie oder die Migration wissenschaftlicher Ideen. Besondere Akzente setzen die Themen „Diversität als neues Paradigma für Integration?" und „Vorspiel der Globalisierung. Zur Emigration deutscher Wissenschaftler 1933 bis 1945".
Die Beiträge sind von herausragenden Experten der jeweiligen Gebiete, u. a. durch die Leopoldina-Mitglieder Markus AFFOLTER, Lorraine DASTON, Wolfgang FRÜHWALD, Michael FROTSCHER, Jörg HACKER, Hans KEPPLER und Otmar WIESTLER, in anspruchsvoller, aber durchaus gut verständlicher Form verfasst.

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A 40 Years Encounter with *Escherichia coli*

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Abstract

We describe the use of intracellular *Enterobacteriaceae*, including enteroinvasive *E. coli*, for the development of virulence-attenuated, live recombinant bacteria as promising agents in tumor therapy. In particular, we present data showing that the type 1 secretion system (T1SS) of *E. coli* α-hemolysin as well as the transfer of DNA and RNA encoding antigens or functional enzymes into mammalian cells by virulence-attenuated intracellular bacteria (e. g. *Salmonella* and enteroinvasive *E. coli*) are interesting tools for a combined immunological and drug therapeutic treatment of tumors. We further show that the knowledge of the specific metabolic processes involved in intracellular replication of these bacteria may help to optimize the construction of virulence-attenuated carrier strains.

Zusammenfassung

In diesem Beitrag berichten wir über die Verwendung von intrazellulären Enterobakterien, einschließlich der enteroinvasiven *E. coli*, zur Entwicklung von Virulenz-attenuierten, lebendenden rekombinanten Bakterien als vielversprechenden Agenzien in der Tumortherapie. Insbesondere werden das Typ-1-Sekretionssystem des *E. coli*-α-Hämolysins sowie die Übertragung von DNA und RNA kodierenden Antigenen oder funktionellen Enzymen in Säugerzellen durch Virulenz-attenuierte intrazelluläre Bakterien (z. B. *Salmonella* und enteroinvasive *E. coli*) beschrieben. Beide Ansätze sind im Hinblick auf eine kombinierte immunologische und medikamentöse Tumortherapie interessant. Darüber hinaus zeigen wir, dass die Kenntnis der metabolischen Vorgänge, die für die intrazelluläre Replikation dieser Bakterien von Bedeutung sind, dazu beitragen kann, die Konstruktion von Virulenz-attenuierten Trägerstämmen zu optimieren.

1. Introduction

Over the past 40 years several aspects concerning metabolism and molecular genetics of *Escherichia coli* were studied in our group. These studies included – among others – characterization of plasmids and virulence factors of extraintestinal *E. coli* strains, such as α-hemolysin (Goebel et al. 1974, Holland 2005). The latter cytolysin is the prototype of a protein that is secreted by a type I secretion system (T1SS). This rather simple protein transport system can be successfully applied for efficiently secreting microbial and tumor

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antigens and thus represents an interesting tool for the development of recombinant live vaccines (Spreng 1999). Some members of the *Enterobacteriaceae*, such as *Salmonella* and *Shigella* (or enteroinvasive *E. coli*, EIEC), are appropriate bacterial carriers for the development of such live vaccines as they induce mainly cellular immune responses, needed for the elimination of intracellular pathogens but also of tumor cells. The latter pathogenic bacteria have to be properly attenuated in virulence in order to be used as carrier systems. Virulence attenuation of intracellular pathogens is optimal when the invasiveness of the bacterial carrier strains into the mammalian cells is retained but its intracellular replication capacity reduced. For the rational design of such virulence-attenuated intracellular bacterial carriers the precise knowledge of the bacterial metabolism inside mammalian host cells is very helpful. In this communication we report on basic studies concerning the presentation of suitable antigens and enzymes by bacterial carriers, their metabolism inside mammalian host cells and bacterial growth within solid tumors in mice. The results may be of importance for the future development of virulence-attenuated *Salmonella*- or EIEC-based recombinant live tumor vaccines.

2. Results

2.1 Secretion of Microbial and Tumor Antigens in Salmonella Typhi Ty21 by T1SS

α-hemolysin is a protein exotoxin which is frequently found in extraintestinal pathogenic *E. coli* strains, particularly in UPEC. The secretion system of this virulence factor represents the prototype of type I secretion systems (T1SS) of Gram-negative bacteria (Holland 2005). It consists of the three proteins HlyB, HlyD and TolC (Wagner et al. 1983, Wandersman and Delepelaire 1990) which form a transport channel that allows the simultaneous, sec-independent protein transport across both membranes of the Gram-negative cell envelope. The secretion signal (HlyAs), essential but also sufficient for transport by this transport system, is located at the carboxy terminal end of α-hemolysin. Vector plasmids have been previously constructed (Gentschev et al. 2002) that allow the easy combination of this secretion signal with any heterologous protein, including microbial and tumor antigens (Gentschev et al. 2005). These recombinant plasmids can be introduced into *Salmonella typhi* Ty21, the vaccine strain against typhoid fever. This strain secretes most of the tested HlyAs-fused antigens with similar efficiency as *E. coli*.

3. Metabolism of Intracellular Bacteria within Mammalian Host Cells

The use of intracellular bacteria such as *Salmonella enterica* (serovar *Typhimurium* or *Typhi*) or *Listeria monocytogenes* as carriers for the development of bacteria-based recombinant live vaccines allows the induction of strong cellular immune responses (Th1 and/or CTL) against the heterologous secreted antigens (Loessner and Weiss 2004). Suitable virulence-attenuated strains are, however, indispensable for the application of these pathogenic bacteria as vaccine carriers. Optimally attenuated strains seem to be those which still allow efficient invasion into and survival within these non-phagocytic host cells but strongly impaired replication within these cells. Such virulence-attenuated strains will still lead to good cellular immune responses.

For this goal we recently studied the metabolism of intracellularly growing *L. monocytogenes* as an intracellular model system applying ^{13}C-isotopologue analysis (EISENREICH et al. 2006). These studies revealed that mainly C3-components deriving from the intermediary catabolic pathways of the host cells and glucose-6-phosphate are major carbon sources for intracellular metabolism of these bacteria (EYLERT et al., manuscript in preparation). Deletion of the genes involved in these catabolic steps indeed lead to virulence attenuation without interfering with invasion and intracellular survival of the *L. monocytogenes* mutants. Recent metabolic studies with *Salmonella enterica* serovar *Typhimurium* showed that neither glucose nor glucose-6-phosphate are used as major carbon sources for intracellular growth. In contrast deletion of genes involved in the uptake of glucose and glucose-6-phosphate lead to a strong impairment of intracellular replication of EIEC.

4. Growth of *E. coli* in Solid Tumors in Mice

It has been repeatedly shown (MENGESHA et al. 2007), that anaerobic or facultative anaerobic bacteria when introduced intravenously into tumor-bearing mice will reach the tumor and multiply within the tumor. We have used a tumor-mouse model with transplanted 4T1 solid tumors to study in detail the fate of *E. coli* K-12 colonizing the tumor after injection of these bacteria into the tail vein and the effect of the bacterial colonization for the tumor. A histological analysis of the colonized tumor shows extensive necrosis of tumor cells and tumor-associated macrophages (TAM) and total destruction of vascularization in the inner section of the tumor which seems to be caused by bacteria-mediated high induction of TNF-α and other proinflammatory components. Massive bacterial colonization occurs in the outer part of this necrotic zone. This zone with growing bacteria is shielded off the peripheral proliferating tumor cells by a line of macrophages which slowly eliminate the bacteria (WEIBEL et al., manuscript submitted). A similar picture was observed when EIEC or *S. enterica* were applied. Thus the bacterial colonization alone despite the initial necrosis of the inner part of the tumor does not lead to the stop of the overall tumor growth. A highly significant reduction in tumor growth was, however, observed when the mice were vaccinated with *S. typhi* Ty21 secreting tumor antigens (as CtxB-fused proteins) via T1SS (GENTSCHEV et al., in press), indicating that tumor-associated T-lymphocytes might be activated by this vaccination.

5. Bactofection of Tumor Cells with Bacterial Carriers Introducing DNA for Prodrug-Converting Enzymes

Recently, we constructed autolysing *L. monocytogenes* carrier strains that are able to introduce c-DNA or RNA encoding functional proteins into the cytosol of mammalian cells (SCHOEN et al. 2004). This method was also successfully applied for introducing into tumor cells (B16 melanoma cells and 4T1 breast tumor cells) DNA, encoding enzymes that convert non-toxic prodrugs, such as 5-fluorocytosine or 6-methylpurine deoxyribose, into the cell-toxic drugs 5-fluorouracil and 6-methylpurine, respectively. It was shown that the tumor cells bactofected with this DNA were completely killed *in vitro* (STRITZKER et al., manuscript submitted). Preliminary data indicate that this procedure is also active in the mouse

tumor model (Stritzker, unpublished observation). The conversion of 6-methylpurine deoxyribose to 6-methyl purine is best achieved by the *E. coli* enzyme purine nucleoside phosphorylase (PNP) encoded by the *deoD* gene. This gene is also present in EIEC strains.

6. Conclusions

The Gram-negative intracellular *Enterobacteriaceae*, especially *Salmonella* and *Shigella/EIEC* are well-suited as carriers for antigen delivery via T1SS. It has been shown that bactofection of mammalian cells can also be achieved by these bacteria (Loessner and Weiss 2004). Especially EIEC strains with optimized virulence attenuation (by blocking specific metabolic reactions essential for intracellular replication) may be well-suited as bacterial carrier systems. These bacteria secrete heterologous antigens directly into the cytosol of antigen-presenting cells which will lead to strong cellular immune responses, including cytotoxic T-cell reponse. Furthermore, EIEC strains synthesize already purine nucleoside phosphorylase (PNP), a potent prodrug-drug converting enzyme, whose production can be further improved and adapted to tumor cells. In addition these bacterial carriers can be loaded with DNA encoding cytotoxic agents that may be directly expressed in the invaded tumor cells.

Thus, enteroinvasive *E. coli* may serve as an excellent tool for a combined bacteria-mediated tumor therapy which includes immune responses as well as cytotoxic drug reactions directed against the tumor cells.

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Genomics and Gene Regulation

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The Avenue to Systemic Infections – Genomics and Beyond

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With 1 Figure and 1 Table

Abstract

Systemic infections require the presence of unique survival genes, enabling the bacteria to cope with the cellular immune system and with the harsh conditions in the serum. Some of the genetic factors which evolved to enable systemic infections are easily identified by genomic analyses. Surprisingly, in *Escherichia coli* strains involved with systemic infections these factors are highly variable. Another unexpected finding is that some of the important virulence-related traits cannot be predicted from sequencing data and bioinformatic analyses.

Zusammenfassung

Systemische Infektionen erfordern die Präsenz von besonderen „Überlebensgenen", die die Bakterien befähigen, mit dem zellulären Immunsystem und den schwierigen Bedingungen im Serum zurechtzukommen. Einige der genetischen Faktoren, die sich im Zuge der systemischen Infektionen entwickelten, können durch Genomanalysen leicht identifiziert werden. Erstaunlicherweise sind diese Faktoren von *Escherichia coli*-Stämmen, die an systemischen Infektionen beteiligt sind, hochvariabel. Eine andere unerwartete Entdeckung war, dass wichtige Virulenz-assoziierte Eigenschaften nicht durch Sequenzdaten und bioinformatische Analysen vorhergesagt werden können.

Septicemic *Escherichia coli* strains constitute an increasing problem for human medicine, especially in patients which are immunocompromised due to disease, chemotherapy or old age. These strains are the leading cause of bloodstream infections in nursing homes, hospitalized persons homes and young children, especially newborns. The problem is highly important especially due to the high incidence of drug resistance often transmissible by plasmids. In recent years it is even more difficult to combat *E. coli* systemic infections due to the high frequency of strains with extended spectrum β-lactamase (ESBL).

Septic *E. coli* strains contain virulence factors which enable them to survive in the host blood and tissues (Mokady et al. 2005a). We conducted genomic analyses of septicemic *E. coli* strains in order to identify the genetic factors that enable the septicemic disease. For this purpose we studied several septicemic strains of serotypes O2 and O78. Using subtractive hybridization studies we obtained about 160 sequences that are unique to the septicemic strains and are not present in non virulent K-12 strains (Mokady et al. 2005b). The unique sequences included many sequences that showed similarity to genes related to virulence. A study of these sequences provided evidence for numerous recombinational events

and horizontal gene transfer leading to a high diversity of virulence factors. This high diversity was not expected, as all the strains are involved in the same disease. However, there were several genetic systems that were shared by all the septicemic strains. These included several iron acquisition systems that were absent from the nonpathogenic strain, most of them, with the exception of yersiniabactin, were coded by the virulence associated plasmid ColV. We sequenced two such ColV plasmids and compared the sequences with two additional plasmids whose sequences are available in the data bases. All the plasmids contain three iron uptake systems and genes for serum resistance.

The results presented in Figure 1 illustrate the genetic map of several ColV plasmids. Table 1 summarizes the virulence-related genes present in ColV plasmids. These genes include several iron uptake systems as well as serum resistance genes.

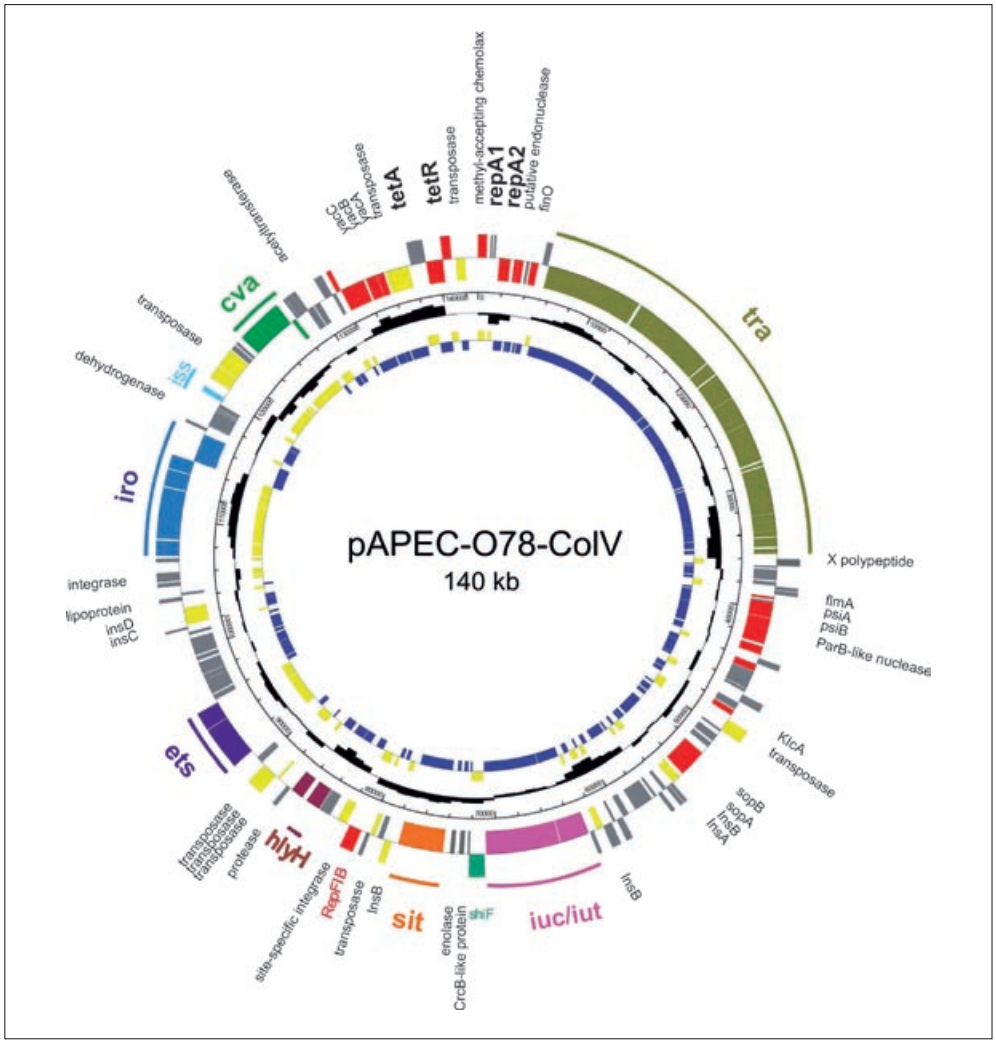

Fig. 1 ColV plasmids of several septicemic *E. coli* strains

Tab. 1 Virulence factors on ColV plasmids

Genes	Operon	Function
iutA, iucD, iutC, iucB, iucA	*iuc/iut*	Aerobactin iron uptake system
sitA, B, C, D	*sit*	Iron binding system
iroB, C, D, E, N	*iro*	Iron binding system salmochelin
iss	–	Serum survival gene involved in complement resistance
etsA, B, C	*ets*	ABC transporter, secretion system
hlyH	–	Hemolysin F protein

These results constitute the genomic basis for understanding the virulence of ExPEC strains. However, based only on genomics one would miss important virulence features. Two such features will be discussed here.

Curli fibers – these are thin aggregative fibers that are present in all the strains of *E. coli.* However, we provide evidence that the curli fibers from a septicemic *E. coli* strain are produced under host conditions – high temperature and high osmolarity – in contrast to the production of curli fibers by non virulent K-12 strains. The mutation appears to be in the *csgD* gene, coding for the regulatory element (Gophna et al. 2001, 2002). The high expression of curli enables internalization of the septicemic *E. coli* strain into mammalian tissue cells.

ETT2 sepsis – virulent *E. coli* strains often contain a Type Three Secretion System (T3SS) that translocates proteins into host cells. Genomic evidence indicated that *E. coli* O157 contains two such systems – the conserved one and an additional system called ETT2 – *E. coli* Type Three 2. This secretion system was also identified in septicemic *E. coli* strain. However, in these strains the ETT2 contains a large deletion and several stop codons, making it impossible to produce the "needle" required for protein translocation. We present data that the ETT2 deletion is conserved in many septicemic strains and therefore called it ETT2 sepsis (Ideses et al. 2005). This secretion system is probably unable to function in secretion, because it is degenerated. Yet, we could show that this degenerated system is essential for virulence.

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Evolution und Menschwerdung

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Evolution und Menschwerdung gehören noch immer zu den interessantesten Themen, mit denen sich die Naturwissenschaft auseinandersetzt und die die Öffentlichkeit faszinieren. Die Thematik verlangt eine interdisziplinäre Auseinandersetzung, für die eine Akademie wie die Leopoldina prädestiniert ist. Daher griff die Jahresversammlung 2005 verschiedene Aspekte hierzu auf.

Die Schwerpunkte der Tagung spiegeln den enormen Fortschritt der Erkenntnisse über das Evolutionsgeschehen und den veränderten Blickwinkel wider, der sich aufgrund des außerordentlich großen Wissenszuwachses und veränderter Diskussionsebenen in der Forschung, aber auch zwischen Wissenschaft und Gesellschaft ergeben. Die Evolution des Menschen und dessen physische, geistige und kulturelle Entwicklungstendenzen stehen dabei im Zentrum.

Der Band spannt den Bogen vom Urknall und der Bildung der Planetensysteme über die Entstehung des Lebens, die Entwicklung von Prokaryoten und Eukaryoten, die Evolution und das Sterben der Saurier, die Analyse von Insektenstaaten bis hin zu Fragen der Menschwerdung und Formen der menschlichen Kultur. Hier werden unter anderem „Das Sprachmosaik und seine Evolution", die „Evolution durch Schrift", Rituale, Religionen, Gemeinschaftsbildung und sozialer Wandel unter evolutionären Aspekten untersucht, aber auch „Bilder in Evolution und Evolutionstheorie" sowie die „Griechischen Anfänge der Wissenschaft" betrachtet.

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Cyclic-di-GMP Signaling in "Life-Style" Switching of *Escherichia coli*

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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). Specific GGDEF/EAL modules control distinct molecular functions in parallel, indicating functional sequestration of c-di-GMP-mediated regulation. Moreover, a detailed model for the coordinated action of several GGDEF/EAL modules in the switch from motility to curli expression during the transition into stationary phase is presented.

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 von vielen dieser Enzyme wird von σ^S (RpoS) aktiviert. Verschiedene GGDEF/EAL-Module regulieren parallel unterschiedliche molekulare Funktionen, was auf eine funktionelle Sequestrierung dieser Module hinweist. Vorgestellt wird auch ein detailliertes Modell für die koordinierte Wirkung verschiedener solcher Module beim Übergang von Motilität zu Curli-Expression während des Eintritts in die stationäre Phase.

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

Bacteria can occur as motile single cells expressing flagella or in surface-associated multicellular communities, i. e. biofilms, where adhesive fimbriae and extracellular matrix are produced. Recently, a novel ubiquitous bacterial signaling molecule, bis-(3'–5')-cyclic-di-guanosine monophosphate (c-di-GMP), was found to play a crucial role in switching between these "life-styles". While c-di-GMP was actually reported about 20 years ago by Moshe Benziman and coworkers as an allosteric activator for the enzyme cellulose synthase (Weinhouse et al. 1997), it was recognized only during recent years that this signal-

ing molecule is ubiquitous in bacteria (but not occurring in archaea or eukaryotes) and plays a key role in the control of various biofilm functions, motility, virulence and developmental processes (for recent reviews, see Jenal and Malone 2006, Römling and Amikam 2006).

C-di-GMP is produced and degraded by diguanylate cyclases (DGC; carrying GGDEF domains, with this amino acid sequence motif representing a loop right in the active centre of the enzymes) and specific phosphodiesterases (PDE; most of which belong to the EAL domain family, with the remaining few featuring HD-GYP domains), respectively. Most of these proteins carry N-terminal sensory input domains, and in a subset, GGDEF and EAL domains are combined in a single polypeptide (Jenal and Malone 2006). Cellular pools of c-di-GMP are believed to be controlled by the opposing activities of DGCs and PDEs, which are in turn controlled by signals that affect their expression and, via the N-terminal sensory input domains, their activities. Most GGDEF proteins also contain an allosteric inhibitory c-di-GMP-binding site (the I-site; approx. K_I = 1 μM), which probably sets a limit to the maximal accumulation of c-di-GMP (Christen et al. 2006).

The molecular c-di-GMP-binding effector components are only beginning to be identified. Such an effector with high c-di-GMP affinity (approx. K_D = 200 nM) is the PilZ domain (Ryjenkov et al. 2006, Christen et al. 2007). The affinities of these c-di-GMP binding sites suggest that the cellular level of this signaling molecule varies somewhere in the higher nM to μM range. In *E. coli*, the PilZ domain occurs only in two proteins, YcgR and BcsA, which control motility and cellulose biosynthesis, respectively. Additional effector candidates are GGDEF proteins themselves, specifically those with the I-site (some of which actually have degenerate GGDEF motifs and therefore cannot be DGCs). Targets directly affected by the various c-di-GMP effector components are mostly elusive, but it is clear that these are very diverse, as they affect transcriptional and perhaps post-transcriptional control, enzymatic activities and the motor function of flagella (Jenal and Malone 2006).

Many bacteria have up to several dozens of GGDEF and EAL proteins (most *E. coli* K-12 strains have 28), which raises questions of signaling specificity. Do these proteins affect different target processes or do their activities all converge into the control of the cytoplasmic pool of c-di-GMP and thereby of a common set of target genes or processes? And if the latter is the case, how can specificity of signaling be achieved with several dozens of GGDEF and EAL proteins? As a solution to this problem, we have suggested sequestration of GGDEF and EAL proteins (Weber et al. 2006): sequestration can be

(*i*) *temporal*, i. e. occur by regulation (only a few proteins should be expressed and be active at the same time);
(*ii*) *functional*, i. e. some of these proteins could occur in complexes together with effector components, in which signaling could be locally confined, and
(*iii*) *spatial*, i. e. such locally signaling complexes may also be located to specific positions in the cell (e. g. to the cell pole, as shown for PleD, a GGDEF protein essential for *Caulobacter* differentiation; Paul et al. 2004).

Thus, sequestration of GGDEF/EAL/effector/target modules in the cell would allow to use this signaling module in a flexible manner at many positions in the cellular regulatory network (Weber et al. 2006).

2. In *Escherichia coli,* c-di-GMP Synthesis and Signaling Is Associated with the σ^S-Mediated General Stress Response and Entry into Stationary Phase

When the almost 500 genes (i. e. about 10 % of the genes in the *E. coli* genome) directly or indirectly activated by the general stress response sigma factor σ^S (RpoS) were identified by microarray analysis (Weber et al. 2005), we noticed that GGDEF/EAL genes seemed to be overrepresented within this group of genes. When regulatory patterns of all 28 GGDEF/EAL genes were determined in detail using single copy *lacZ* reporter fusions, 21 of these genes were found to be expressed in rich medium, with 10 being under positive and 5 being under negative control of σ^S. As the σ^S-activated genes are all induced during entry into stationary phase (Sommerfeldt et al. 2008), these findings strongly indicate that at least in *E. coli*, c-di-GMP signaling mainly coordinates processes during entry into stationary phase.

3. The GGDEF/EAL Module YdaM/YciR Specifically Controls the Expression of Adhesive Curli Fimbriae

What is the link between σ^S, stationary phase, c-di-GMP and biofilm formation? Already in 1993, it was reported that the expression of thin-aggregative fimbriae, also termed curli, was under the control of σ^S (Olsén et al. 1993). Since then, curli have been recognized as crucial for the formation of biofilms on solid liquid-air interfaces (Römling 2005). Moreover, their expression in *Salmonella* is sensitive to modulation of c-di-GMP levels as can be clearly shown by overproducing certain DGCs or PDEs (Simm et al. 2004).

Using knockout mutations in σ^S-controlled GGDEF/EAL genes it was therefore tested, whether any of these genes is physiologically involved in the control of curli expression (Weber et al. 2006). This is clearly the case for the GGDEF gene *ydaM*, which is required for curli formation, whereas curli fibres are overproduced in mutants deficient for the GGDEF+EAL gene *yciR*. The purified YdaM and YciR proteins are active as a DGC and a PDE, respectively. The target of YdaM/YciR-mediated c-di-GMP control is the transcription of the regulatory gene *csgD*, which codes for an essential activator for the *csgBAC* operon (encoding curli subunits) and for *yaiC*, which codes for another strictly σ^S-dependent GGDEF protein essential for activating cellulose synthase. Genome-wide transcriptional profiling on microarrays demonstrated that the YdaM/YciR c-di-GMP control module affects this system only (Weber et al. 2006), and does not "cross-talk" into the control of motility, which during the same phase of the growth cycle becomes inhibited by another GGDEF/EAL module (see below). This high specificity for *csgD* transcriptional control only suggests that the YdaM/YciR module acts in a local or "micro-compartimented" way.

4. Inverse Coordination of Motility and Curli-Mediated Adhesion by the GGDEF/EAL Module YegE/YhjH

In general, high levels of c-di-GMP up-regulate biofilm functions such as curli-dependent autoaggregation and down-regulate motility (Simm et al. 2004). Based on the simple assumption that being auto-aggregative would be counterproductive for single-cellular mo-

tility and, on the other hand, force-generating flagella should be inactivated in order to allow "settling down" at a surface, one can predict that at the molecular level, motility and curli expression should be mutually exclusive. This means that these functions should be inversely coordinated in a switch-like manner, and suggests that c-di-GMP is a key component in operating this switch. A convenient way to generate such an inverse switch (and potentially bistability) would be to endow each control system, i. e. the regulatory cascades controlling motility and curli expression, with at least one component that inhibits the activity of the other system.

We have recently shown that the motility control cascade with FlhDC as the master regulator contains at least two such components that "cross-talk" into the σ^S/curli control cascade (Pesavento et al. 2008):

(*i*) The first system operates at the level of the master regulators, i. e. FlhDC and the sigma subunits of RNAP, σ^{70}, σ^F (also termed FliA, the flagellar sigma) and σ^S. The key component is the FliZ protein, a class II gene product under direct control of the master regulator FlhDC. FliZ does not affect σ^S levels, but strongly interferes with σ^S activity by a mechanism not yet understood; it thereby down-regulates the expression of many σ^S-controlled genes, indicating that activity of the flagellar expression cascade via FliZ interferes with the activation of the entire general stress response (including curli expression).

(*ii*) Operating at a lower level, the second system relies on the σ^F-controlled, i. e. flagellar class III gene product YhjH, a highly active PDE, which maintains a low level of c-di-GMP, which would otherwise, via the effector protein YcgR, interfere with the motor function of flagella, and, via a still unknown effector component, activate curli expression.

The latter system can be antagonized by two σ^S-induced GGDEF proteins, YegE (which acts as the major player) and YedQ, which thereby promote inhibition of flagellar activity and curli induction. Switching from motility to curli expression (which happens during a precise time point during entry into stationary phase) requires a shift in the balance between the EAL protein YhjH and the GGDEF proteins YegE and YedQ. This shift is driven by two processes (Pesavento et al. 2008):

(*i*) a precise down-regulation of flagellar gene expression (at all levels of the cascade), which does also essentially involve proteolytic degradation of the master regulator FlhDC (Tomoyasu et al. 2003) and excess flagellar σ^F not bound by its anti-sigma FlgM (Barembruch and Hengge 2007) by the complex ATP-driven proteases ClpXP and Lon, respectively, and

(*ii*) accumulation and activation (i. e. successful competition for RNAP core) of σ^S during entry into stationary phase.

Taken together, the YegE/YhjH c-di-GMP control module emerges as a *checkpoint system* that monitors, when the motility expression system has been shut down, which is reflected by YhjH-mediated PDE activity having fallen below the threshold that allows c-di-GMP accumulation driven by the GGDEF proteins that are induced in parallel. Once this has happened, existing flagella are inhibited (via YcgR) and curli expression is activated. Interestingly, the latter process involves two c-di-GMP control modules, i. e. YdaM/YciR and YegE/YhjH. Although both systems affect the exceedingly complex transcriptional control of the curli activator gene *csgD*, all genetic evidence available to date indicates that

(*i*) these systems do not act in a redundant and additive way, but are both required, perhaps in a sequential manner; and
(*ii*) while the YegE/YhjH system controls the cytoplasmic c-di-GMP pool, the YdaM/YciR system acts in a highly specific local manner (Pesavento et al. 2008).

5. Conclusions and Perspectives

Until recently, high c-di-GMP levels were only generally known to promote adhesion and biofilm formation and to interfere with motility. Based on new results of our and other groups, we now begin to understand the molecular details of this inverse control, in particular the precise molecular effects of distinct GGDEF/EAL proteins. Evidence is also accumulating that the concept of temporal, functional and even spatial sequestration of different c-di-GMP control modules is correct, with some GGDEF/EAL proteins obviously controlling the cellular pool of freely diffusible c-di-GMP and thereby affecting several target functions, and others acting in a highly local "microcompartimented" manner on one specific target only. Moreover, the physiological conditions of c-di-GMP control are becoming clear: In *E. coli*, c-di-GMP control modules orchestrate the complex sequence of events that lead from rapid unlimited growth to a restricted growth situation, in which the cells become highly motile and use a "foraging strategy", followed by entry into stationary phase, where motility is "given up" in favour of biofilm formation.

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The Hha/YdgT Proteins Participate in H-NS-Mediated Silencing of Horizontally-Acquired DNA in Enteric Bacteria

Aitziber Vivero[1], Rosa C. Baños[2], Cristina Madrid[1], and Antonio Juárez[1,2]

With 1 Figure

Abstract

A relevant role for the H-NS protein is to silence xenogeneic DNA. Protein-protein interaction studies have shown that Hha-like proteins interact with members of the H-NS family of proteins and modulate gene expression. Transcriptomic data about the effect of the depletion of Hha-like proteins in *Salmonella enterica* serovar Typhimurium provides evidence that main targets for Hha and its paralogue YdgT are those genes encoded in AT-rich horizontally acquired DNA sequences. Hence, Hha and YdgT must form complexes with H-NS when silencing these DNA regions.

Zusammenfassung

Eine wichtige Funktion des H-NS-Proteins ist seine Fähigkeit, xenogenetische DNA auszuschalten. Protein-Protein-Interaktionsstudien zeigten, dass Hha-ähnliche Proteine mit Mitgliedern der H-NS-Familie in Wechselwirkung treten und die Genexpression modulieren. Die Ergebnisse von Transkriptionsanalysen belegen, dass der Effekt der Reduktion von Hha-ähnlichen Proteinen in *Salmonella enterica* Serovar Typhimurium darauf zurückzuführen ist, dass die wichtigsten Zielstrukturen für Hha und sein Paralog YdgT diejenigen Gene sind, die in AT-reichen, durch horizontalen Gentransfer erworbenen DNA-Bereichen kodiert werden.

1. Introduction

The genomes of all genera from the family *Enterobacteriaceae* contain at least one copy of a gene that encodes a member of the Hha-YmoA family of proteins (Madrid et al. 2007). These low-molecular-mass proteins (about 8.6 kDa) have initially been identified as modulators of the expression of virulence factors, such as the *E. coli* toxin α-haemolysin (the Hha protein) or the *Y. enterocolitica* Yop proteins and YadA adhesin (the YmoA protein) (Cornelis et al. 1991, Nieto et al. 2002). Cells depleted of Hha exhibit phenotypic properties similar to those of cells lacking the nucleoid-associated protein (NAP) H-NS, and it was proposed that Hha-like proteins represented a new class of NAPs.

Several lines of evidence have shown that proteins of the Hha family interact with members of the H-NS family to modulate gene expression (Madrid et al. 2002, Nieto et al.

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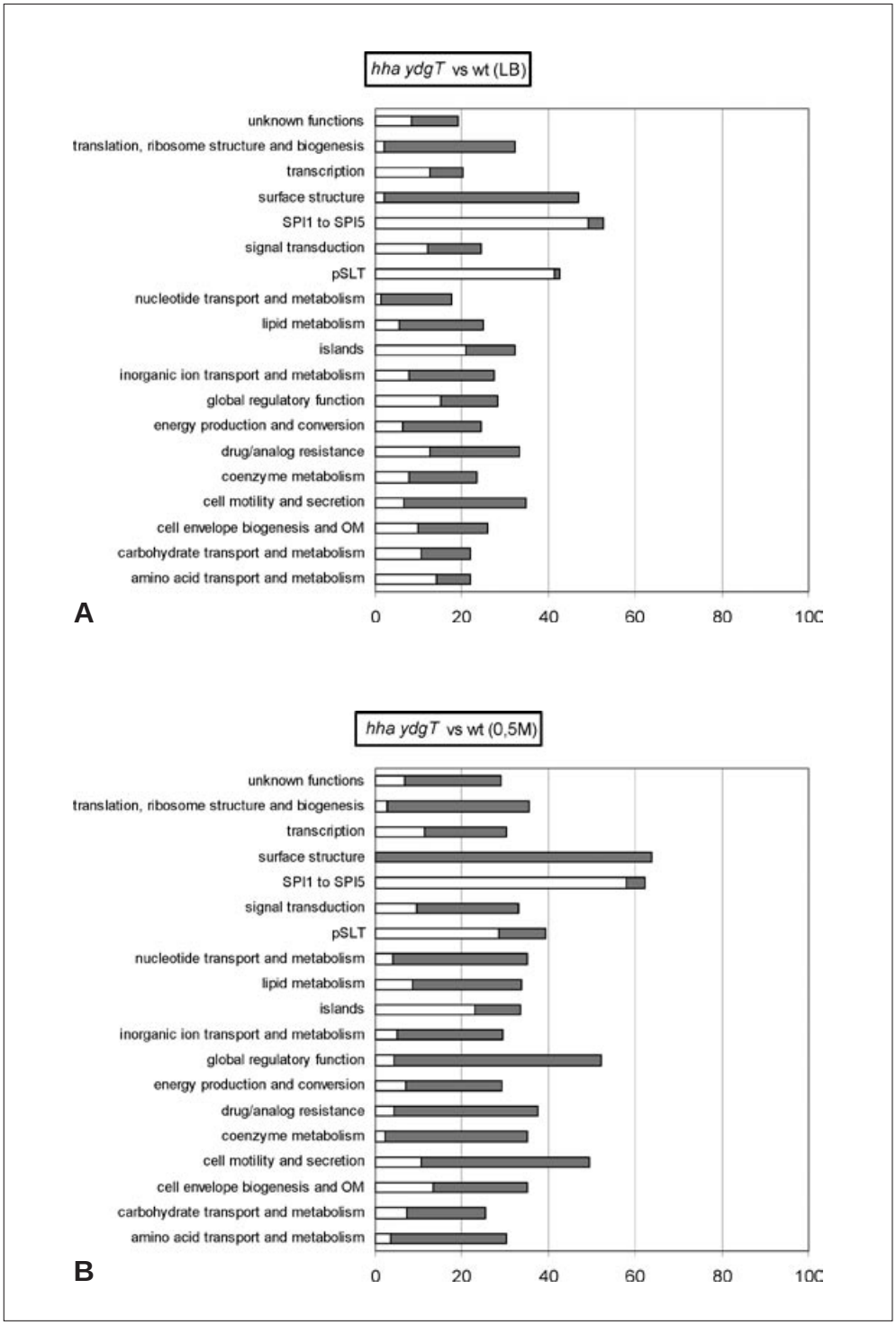
hha ydgT vs wt (LB)
unknown functions
translation, ribosome structure and biogenesis
transcription
surface structure
SPI1 to SPI5
signal transduction
pSLT
nucleotide transport and metabolism
lipid metabolism
islands
inorganic ion transport and metabolism
global regulatory function
energy production and conversion
drug/analog resistance
coenzyme metabolism
cell motility and secretion
cell envelope biogenesis and OM
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unknown functions
translation, ribosome structure and biogenesis
transcription
surface structure
SPI1 to SPI5
signal transduction
pSLT
nucleotide transport and metabolism
lipid metabolism
islands
inorganic ion transport and metabolism
global regulatory function
energy production and conversion
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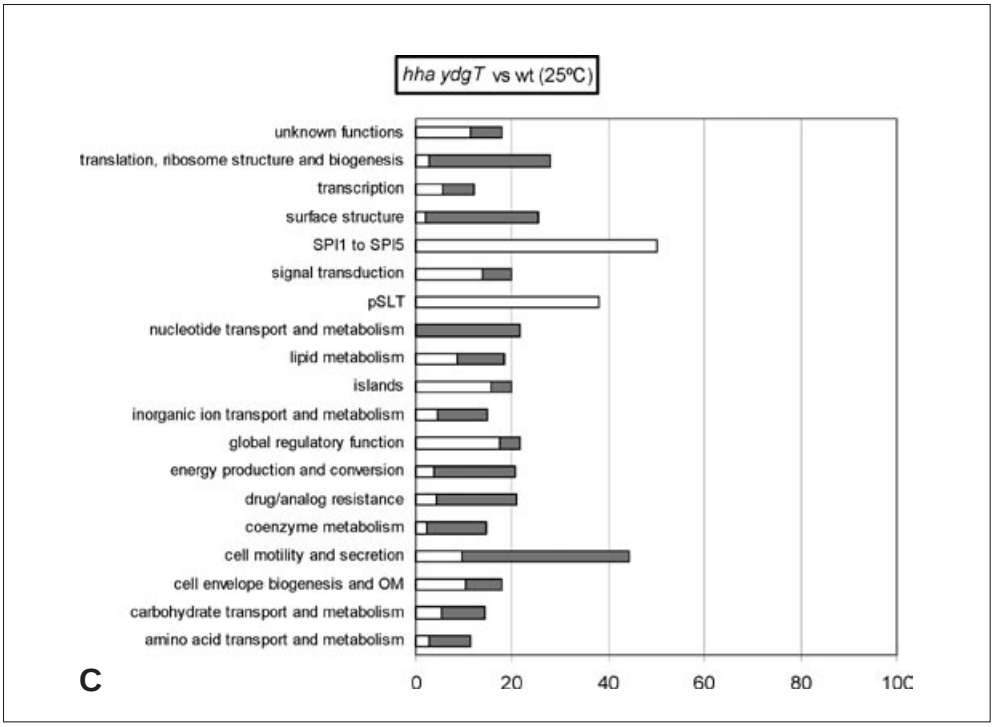

Fig. 1 Expression changes of genes belonging to functional groups and pathogenicity islands obtained at three different growth conditions: (A) LB at 37 ºC, (B) LB 0,5 M NaCl at 37 ºC and (C) LB at 25 ºC. The bars show the percentage of genes belonging to each group that show altered expression in hha ydgT cells. The grey bars indicate the proportion of genes that are down-regulated ($M < 0$), and the white bars represent the proportion of up-regulated genes for each group ($M > 0$).

2000). Hha-like proteins show structural mimicry to the H-NS oligomerization domain and interaction of Hha with H-NS increases the repressory ability of this latter protein (Madrid et al. 2002, 2007, Nieto et al. 2002). H-NS is the most extensively studied example of a NAP that participates in environmentally-dependent modulation of gene expression (Dorman 2004). H-NS is considered as a transcriptional repressor, playing a relevant role silencing xenogeneic DNA (Lucchini et al. 2006, Navarre et al. 2006, Pflum 2006).

It remained to be determined whether the set of H-NS-regulated genes coincides with the set of Hha-regulated genes, or whether the latter is simply a subset of the former. Recently obtained transcriptomic data show that main targets for Hha and/or its paralogue YdgT are those genes encoded in horizontally acquired DNA sequences that are silenced by H-NS.

2. Results

The genome of *Salmonella* contains, in addition to *hha*, a copy of the *ydgT* gene, which codes for an Hha paralogue, YdgT. To avoid the attenuation of an *hha* mutant by overexpression YdgT, the transcriptomic analysis was done in a *S.* Typhimurium *hha ydgT* double

mutant strain (SV5015HY). Microarray data showed that when compared to wt strain, strain SV5015HY exhibited altered expression of about 1,000 genes. The mRNA levels of 471 genes were > 2-fold higher in the *hha ydgT* mutant, indicating that Hha/YdgT repress gene expression in the wt strain. The mRNA levels of 504 genes were > 2-fold lower in the mutant strain, indicating that Hha/YdgT activate gene expression in the wt strain. Up-regulation predominated in genes belonging to several functional categories, including genomic islands (SPI1 to SPI5 and other genes related to horizontally acquired DNA regions), and the pSLT plasmid. In contrast, down-regulation predominated in genes belonging to functional categories of surface structure, cell motility-secretion, and translation (Vivero et al. 2008).

Most of the genes belonging to pathogenicity islands SPI1 to SPI5 (93.22 % of the genes) are predominantly over-expressed in strain SV5015HY. This leads to virulence attenuation, as shown when the competitive index is measured. When compared to the wt strain, a double *hha ydgT* mutant shows a clear virulence attenuation.

Another category of genes showing altered expression in cells lacking Hha/YdgT proteins corresponds to ORFs encoded in the virulence plasmid pSLT. More than 40 % presented an altered expression in strain SV5015HY. Most of them (97 %) were over-expressed, including those from the *tra* operon. To test the effect of *hha ydgT* mutations in the conjugation frequency of plasmid pSLT, mating experiments were performed. When compared to the wt strain, the frequency of pSLT plasmid transfer was 10-fold higher when double mutant (*hha ydgT*) strain was used as donor (1.84×10^{-5} and 1.75×10^{-4} respectively).

Genes showing a reduced expression in strain SV5015HY belong to several functional categories. The decreased expression of many of these genes may be due to an indirect effect of the double mutation on cell physiology (most likely those belonging to the functional category of translation, ribosome structure and biogenesis) or on specific transcriptional repressors. Remarkably, a significant set of genes showing reduced expression includes many involved in flagellar biogenesis. This results in a reduced motility, as shown when bacterial growth halos of SV5015 (wt) and SV5015HY (*hha ydgT*) strains in motility agar plates were compared. Strain SV5015HY forms a very small halo. Transformation of strain SV5015HY with a plasmid that codifies the *hha* gene (pUBM22) complemented the low-motility phenotype.

Above referred results were obtained from exponentially growing cells in LB medium at 37 ºC. Remarkably, when either the growth temperature was lowered (25 ºC) or the medium osmolarity was shifted to LB 0.5 M NaCl, the set of deregulated genes in strain SV5015HY was very similar (Fig. 1).

Hha-like proteins form nucleoprotein complexes with H-NS. Recent results presented independently by different groups have shown that this NAP silences the expression of horizontally acquired DNA under non-permissive conditions (Navarre et al. 2006, Pflum 2006). Hence a significant set of genes that are silenced by H-NS should also be silenced by Hha/YdgT. To confirm this, we compared the reported H-NS binding sites within the *Salmonella* genome (Navarre et al. 2006) and our transcriptomic data. The results clearly support the hypothesis that Hha/YdgT interact with H-NS to favor silencing of xenogeneic DNA. 87 % of SPIs genes repressed by Hha/YdgT have been reported to contain H-NS binding sites. The coincidence is as well high for other SV5015HY up-regulated genes belonging to other genomic islands (34 %) and for the pSLT plasmid (46 %). It has been suggested that H-NS is not an effective silencer at all binding sites for some horizontally trans-

ferred sequences (Navarre et al. 2006). This is supported by the finding that Hha-like proteins also participate in silencing xenogeneic DNA.

3. Conclusions

The transcriptomic analysis of a *S.* Thypimurium *hha ydgT* double mutant shows altered expression of about 1,000 genes (471 induced and 504 repressed). The change of gene expression pattern can be related to detectable phenotypes: an attenuated virulence, reduced motility and an abnormally high conjugation frequency. Three functional categories include genes predominantly showing an induced expression: genes mapping in SPIs 1 to 5, in other genomic islands, and in pSLT plasmid. Hence, Hha like proteins appear to have evolved to negatively modulate expression of horizontally-acquired genes in enteric bacteria.

The data obtained with strain SV5015HY support the hypothesis that Hha/YdgT interact with H-NS to favor silencing of xenogeneic DNA.

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Regulation of Virulence Genes in *Shigella* Hovers between Gain and Loss of Transcriptional Activators

Gianni Prosseda[1], Mariassunta Casalino[2], and Bianca Colonna[1]

With 1 Figure

Abstract

The evolution of *Escherichia coli* towards pathogenicity represents an interesting model because of the enormous versatility of this microorganism, causing an impressive variety of different diseases. Comparative genomic analysis has shown that *Shigella*, rather than being a genus with four species, belongs to the highly diversified *Escherichia coli* species. The transition of *Shigella* towards a pathogenic phenotype offers major clues for understanding the steps triggering a virulence phenotype in a commensal organism. Two opposite mechanisms could account for this phenomenon: the acquisition of additional genes encoding virulence determinants by horizontal gene transfer and the loss of modification of pre-existing genetic material.

Zusammenfassung

Die Entwicklung von *Escherichia coli* in Richtung Pathogenität stellt aufgrund der enormen Vielfältigkeit dieses Mikroorganismus, der eine beeindruckende Vielzahl von verschiedenen Krankheiten auslösen kann, ein interessantes Modell dar. Vergleichende Genomanalysen zeigten, dass *Shigella* keine Gattung mit vier verschiedenen Spezies darstellt, sondern eher der höchst breit gefächerten Spezies *Escherichia coli* angehört. Der Übergang von Shigellen hin zu einem pathogenen Phänotyp gibt wichtige Hinweise darauf, welche Schritte in einem kommensalen Organismus nötig sind, um in einen virulenten Phänotyp überführt zu werden. Zwei gegensätzliche Mechanismen könnten für dieses Phänomen in Betracht gezogen werden: Die Aufnahme von zusätzlichen, virulenz-assoziierten Genen durch horizontalen Gentransfer und der Verlust der Fähigkeit bereits vorhandenes genetisches Material zu ändern.

1. Evolution of *Shigella* from *Escherichia coli*

Few microorganisms are as resourceful as *Escherichia coli*. More than being just a harmless intestinal inhabitant, *E. coli* can be a highly versatile, and frequently deadly, pathogen. Recent studies have demonstrated that *Shigella*, which was considered a genus with four species, actually belongs to the extremely diversified *E. coli* "world" and, therefore, should be considered as a pathogenic *E. coli* (Lan and Reeves 2002). As a matter of fact the genomes of *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* are colinear with the *E. coli* one and

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more than 90% homologous to it. *Shigella* is a facultative intracellular pathogen causing a severe enteric syndrome in humans, mainly in the developing countries (Sansonetti 2001). The pathogenicity mechanism of *Shigella* is based on the capacity to reach and invade colonic epithelial cells, multiply intracellularly and spread to adjacent cells with consequent cell death and destruction of the colonic mucosa. This process is highly complex and requires the coordinated expression of virulence factors, encoded not only by chromosomal genes but also by the plasmid genome.

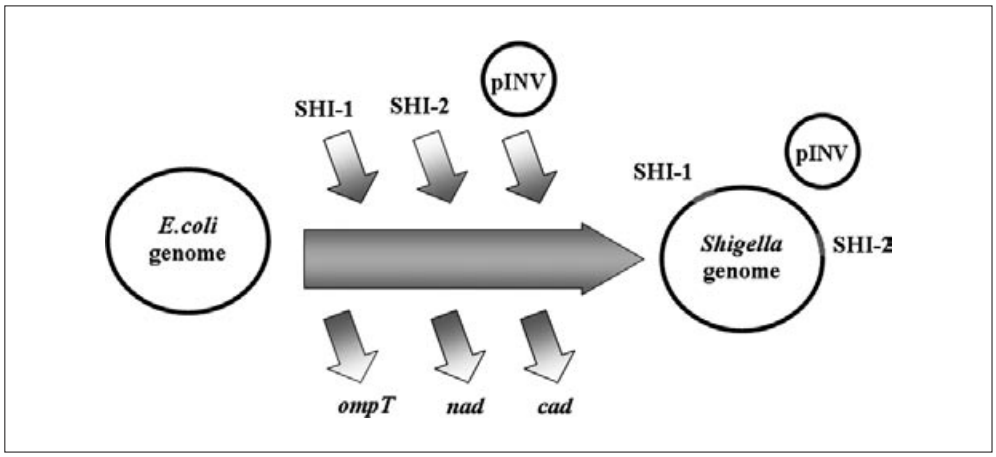

Fig. 1 Evolution of *Shigella* from an ancestral commensal *Escherichia coli*. Acquisition of the large virulence plasmid (pINV) and PAIs (SHI-1 and SHI-2) by horizontal gene transfer is counterbalanced by the loss of genetic systems like *cad*, *nad* and *ompT* whose products are detrimental for the full expression of the invasivity process.

The transition of *Shigella* towards a pathogenic phenotype is a good model for understanding how virulence phenotype is triggered in a commensal organism. Major mechanisms possibly accounting for this phenomenon are the acquisition of additional genes encoding virulence determinants and the inactivation or loss of pre-existing genes.

The acquisition of the virulence plasmid (pINV) has been undoubtedly one of the most critical events in the evolution of the pathogenic lifestyle of *Shigella*. This large plasmid contains all the genes required for expressing the hallmarks of *Shigella* virulence: invasion, intracellular replication, intercellular spread, and induction of inflammatory response. Besides pINV, *Shigella* harbors on its chromosome several pathogenicity islands containing genes involved in the pathogenicity process. In *S. flexneri* the SHI-I pathogenicity island encodes the ShET1 enterotoxin, a cytopathic protease, and a mucinase. The SHI-2 pathogenicity island encodes an aerobactin iron transport system and immunity to several colicins. The acquisition of multiple linked virulence traits by horizontal gene transfer is crucial in triggering a virulence phenotype in a commensal organism (Dobrindt et al. 2004). In the novel habitat the new pathogen then reaches optimal fitness by modifications of its genome which often redesign important metabolic properties.

The lack of several catabolic pathways, like those of lactose or mucate, or the loss of the ability to synthesize flagella or curli could be attributed to a process of niche adaptation. In this context it is worth mentioning that IS sequences play a crucial role in the evolution

of bacterial pathogens either directly, by modifying gene expression through deletions or insertions, or indirectly, by promoting homologous recombination. In *Shigella* the lack of motility has been correlated with insertion of IS1 elements which have determined the appearance of progressive genetic defects, resulting in the complete loss of flagella. Similarly, multiple IS elements have interrupted genes encoding for thin aggregative fimbriae, known as curli, triggering genetic rearrangements resulting in deletions with different extensions in the curli encoding operons.

2. The Main Pathoadaptive Mutations in *Shigella*

Besides the loss of traits important for the survival in the environment but redundant for the life inside the host, the new pathogen may also undergo a series of convergent mutations in genes which negatively interfere with the expression or function of virulence factors required for the survival within the host (Dobrindt et al. 2004). These so called pathoadaptive mutations improve survival within host tissues, increase the pathogenic potential of the new pathogen and drive the evolution of a microorganism towards a more pathogenic phenotype.

While there are several examples of pathogen evolution through acquisition of genes by mobile genetic elements like phages, plasmids or PAIs, the contribution of gene loss to pathogen evolution is only beginning to be hold in due consideration (reviewed in Maurelli 2007, Prosseda et al. 2007). So far in *Shigella* three distinct genetic systems can be considered as antivirulence genes, since their lack has been related to an optimization of the pathogenicity process: the *cad* operon encoding for the lysine decarboxylase system, the *ompT* gene coding for an outer membrane protease and the *nadBA* genes involved in the synthesis of nicotinic acid.

The *ompT* gene is contained within a remnant of a cryptic prophage located on the *E. coli* K-12 genome, and encodes the surface protease OmpT. In *Shigella*, OmpT is able to degrade IcsA (known also as VirG), a pINV encoded protein that is required for intra- and inter-cellular motility. Following the precise excision of a large fragment carrying the *ompT* gene within the remnants of the DLP12 lamboid phage, all *Shigella* strains fail to produce the OmpT protease (Nakata et al. 1993). The *ompT* gene meets the definition of an antivirulence gene, since its introduction in *Shigella* negatively interferes with the invasivity process by reducing the *Shigella* intra- and inter-cellular spreading.

Unlike most *E. coli* strains, *Shigella* is a nicotinic acid auxotroph in that the bacterium is unable to synthesize NAD from exogenous nicotinic acid. In *S. flexneri* this defect is due to alterations in the *nadA* and/or *nadB* genes which encode the enzyme complex that converts L-aspartate to quinolate, a precursor to NAD synthesis. Quinolate is a potent, small molecule able to inhibit several virulence phenotypes of *Shigella*. It is able to block invasion and cell-to-cell spread of *S. flexneri* and also reduces the induction of the transepithelial migration of polymorphonuclear leukocytes (Prunier et al. 2007). All together these results indicate that the auxotrophic requirement for nicotinic acid in *Shigella* represents another example of pathoadaptation through selection by means of loss of a gene function that is incompatible with virulence.

The best known example of pathoadaptive mutation by loss of antivirulence genes in *Shigella* is the case of the *cad* system (Maurelli et al. 1998, Day et al. 2001, Casalino et al.

2003, 2005). All *Shigella* and also enteroinvasive *E. coli*, a group of *E. coli* which shares with *Shigella* the same pathogenicity process, are characterized by the inability to catabolize lysine due to the lack of lysine decarboxylase activity (the LDC^- phenotype). In enteric bacteria low pH induces a programmed molecular response which, among other events, triggers the transcription of several amino acid decarboxylase systems, which in turn mediate maintenance of pH at a level suitable for cell survival. One of the three major amino acid decarboxylase systems is based on the activity of the lysine decarboxylase. In *E. coli* this activity is determined by the products of the *cadBA* operon which encodes lysine decarboxylase (*cadA*) and a lysine-cadaverine antiporter (*cadB*), and is co-induced by low pH and lysine. Expression of the *cadBA* operon is dependent on CadC, a positive activator whose gene maps upstream of the operon and is transcribed independently from the same strand.

Despite the fact that the *cad* system may be important during the bacterial transit through the intestinal tract, in *Shigella* and EIEC such a system has been completely lost. Interestingly, it has been shown that cadaverine, a polyamine resulting from the decarboxylation of lysine, negatively interferes with the *Shigella* pathogenicity process. Cadaverine seems to induce the attenuation of enterotoxicity and also to inhibit the migration of polymorphonuclear leukocytes (PMN) across the intestinal epithelial monolayers. In addition it has been demonstrated that cadaverine reduces the ability of *Shigella* to lyse the phagocyte vacuole, thus preventing the bacterium from interacting with the cytoskeleton. On the basis of this observation it has been postulated that compartimentalization of *Shigella* into a phagolysosome reduces the ability to disseminate intra- and inter-cellularly and perturbs signaling pathways essential to the promotion of PMN transepithelial migration. It has been proposed that silencing of the *cad* locus in *Shigella* represents an important pathoadaptive mutation necessary for increasing the pathogenic potential of bacteria in host tissues (Maurelli et al. 1998, Maurelli 2007).

3. Acquisition and Loss of Transcriptional Activators

The acquisition of the large virulence plasmid (pINV) is the crucial event in the evolution of *Shigella* from non pathogenic commensal *E. coli*. The pINV plasmid contains all the genes required for invasion and for intra- and inter-cellular spread, including their positive activators VirF and VirB (Prosseda et al. 2002). To obtain a proper sensing of the host environment and an adequate virulence response, the expression of virulence determinants is integrated in layers of iterative regulative networks, ensuring a coordinated and on time synthesis of all determinants necessary to induce invasivity. Like in other life-threatening human pathogens, also in *Shigella* the entry from the outer environment into the warmer host milieu is one of the crucial events triggering the expression of virulence factors. The plasmid regulatory cascade of *Shigella* represents an interesting system to understand how pathogenic bacteria have evolved to prevent wasteful expression of virulence factors while ensuring strong and appropriate activation when this is required. The arrival of additional regulators in the novel pathogen through the acquisition of the pINV might have altered the transcriptional program of the ancestral *E. coli* cell. Transcriptomic analysis is currently employed to investigate on what *E. coli* genes are up- or down-regulated in response to an increasing level of the VirF protein, which is the first positive activator of the *Shigella* invasivity cascade (Prosseda et al. 2002).

The acquisition of new positive activators encoded by the pINV is counterbalanced by the loss of some ancestral regulators. Comparative analysis of molecular rearrangements inducing silencing of the *cad* operon in *Shigella* and EIEC suggests that the lack of lysine decarboxylase activity has been attained through diverse strategies (Day et al. 2001, Casalino et al. 2003, 2005). It has been proposed that the first step in the silencing of the cad locus might have been inactivation of the *cadC* gene, followed by a spread of insertion sequences in *cadB* and *cadA*, inducing deletions within the *cad* locus or extending to the flanking regions (Casalino et al. 2003). The observed lack or inactivation of the *cadC* gene can be accounted for by considering that CadC, the regulator of the *cadBA* operon, might play additional roles in controlling cellular functions not directly related to lysine utilization. In order to understand whether the CadC regulator may play a direct or indirect role in the control of other genes involved in the adaptation of the bacterium to the host environment we are currently investigating on how the expression profile of *E. coli* has been affected by the disappearence of the the CadC regulator.

4. Conclusion

In the evolutionary route of *Shigella* from *E. coli* ancestors towards a pathogenic lifestyle, the critical event has been the acquisition of the pINV plasmid through horizontal transfer and the inactivation of pre-existing genes. Since both, the loss and the acquisition of new genetic systems, are flanked by the arrival or by the lack of transcriptional regulators, it is possible to envisage that the transcriptional profile of the ancestor cell has been modified. The identification of such changes can lead to a better understanding of how a commensal bacterium has become a pathogen.

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Identification of the Hha-Binding Motif in the N-Terminal Domain of H-NS

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With 1 Figure and 1 Table

Abstract

In enteric bacteria, the nucleoid associated proteins H-NS and Hha form a regulatory complex that is involved in the modulation of transcription of many environmentally regulated genes. We have used a combination of mutagenesis and Nuclear Magnetic Resonance (NMR) to study the H-NS/Hha interaction. The interaction involves a conformational change of Hha. In H-NS, the Hha binding motif is located in the N-terminal part of the H-NS dimerization domain. The H-NS $Arg^{12}His$ mutation abolished Hha binding *in vitro*, indicating that this arginine is essential for the interaction. In addition, replacement of Asn^{9} for Leu causes a drastic decrease in the binding affinity.

Zusammenfassung

In Enterobakterien bilden die Nucleoid-assoziierten Proteine H-NS und Hha einen regulatorischen Komplex, der in die Modulation der Transkription von vielen, durch Umwelteinflüsse regulierten Gene eingreift. Mit Hilfe einer Kombination aus Mutagenese und Kernspinresonanz (NMR) haben wir die Interaktion zwischen H-NS und Hha untersucht. Die Interaktion beinhaltet eine Konformationsänderung von Hha. Das Hha-Bindungsmotiv befindet sich im H-NS-Protein im N-terminalen Bereich der H-NS-Dimerisierungsdomäne. Dass durch eine $Arg^{12}His$-Mutation im H-NS-Protein *in vitro* die Hha-Bindung unterbunden werden kann, weist darauf hin, dass Arginin für die Interaktion essentiell ist. Darüber hinaus zeigte sich, dass das Ersetzen von Asn^{9} durch Leu eine drastische Reduktion der Bindungsaffinität bewirkt.

1. State of the Art

The nucleoid-associated protein H-NS acts as a negative regulator of gene transcription of a large number of promoters, many of which are regulated in response to environmental changes. H-NS binds preferentially to AT-rich regions of curved DNA promoting further oligomerization of the protein on the DNA molecule. Although the molecular basis for regulation of gene expression is not known, it was suggested that the resulting nucleoprotein complex represses gene transcription by interfering with the RNA polymerase access to specific promoters and/or by blocking transcription elongation (for a recent review see

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Dorman 2007). At least for some genes, H-NS regulation of expression requires the participation of additional proteins that interact with H-NS modifying its modulatory properties. H-NS forms heteromeric complexes with its paralogue, StpA (Johansson et al. 1991), and with members of the Hha family of proteins (Madrid et al. 2007). The best characterized members of this family are the *Escherichia coli* protein Hha and the *Yersinia enterocolitica* protein YmoA. These proteins, which have 82 % sequence identity, modulate the expression of virulence factors in response to environmental factors such as osmolarity or temperature. Other members of the family have been identified in enteric species, observing that several *hha*-like genes could be found in some chromosomes. In addition, Hha proteins may be encoded in various large conjugative plasmids that belong to different incompatibility groups (Madrid et al. 2007).

Studies, focused to understand the regulation of the *E. coli hly* operon, which encodes the α-haemolysin toxin, showed that, rather than exhibiting DNA-binding activity, Hha interacts with H-NS and strengthens the H-NS ability to repress the *hly* operon under non-permissive conditions (Nieto et al. 2000).

Further studies have shown direct interactions between various members of the H-NS and Hha families of proteins. The complexes formed participate in transcription regulation of specific genes required for bacterial pathogenesis (Madrid et al. 2007). However, the lack of molecular details of the process hinders the elucidation of the regulation mechanism. To address this relevant issue, we decided to study the H-NS/Hha interaction using biophysical techniques.

The H-NS protein consists of an N-terminal dimerization domain and a C-terminal DNA-binding domain separated by a flexible linker that is involved in homooligomerization. A complete, high-resolution structure of H-NS has proven elusive, but the three dimensional structures of polypeptides containing the first 46 (Bloch et al. 2003) and 57 (Esposito et al. 2002) H-NS residues have been solved by NMR, observing, in both cases, the formation of a homodimer structure. The three-dimensional structure of the 72-residue protein Hha was solved by NMR (Yee et al. 2002).

The interaction of full length H-NS with Hha was first shown by copurification of the two proteins (Nieto et al. 2000). Recent work from our group demonstrated that the N-terminal domain of H-NS was sufficient for Hha binding (Garcia et al. 2005), while no interaction was observed between Hha and the C-terminal domain of H-NS. The interaction of Hha with the N-terminal domain of H-NS was studied by fluorescence anisotropy and NMR techniques. Titrations of a ^{15}N-labeled Hha sample with a truncated form of the H-NS protein corresponding to residues 1 to 64 (H-NS$_{64}$), followed by NMR, showed that the interaction between both proteins involved a substantial conformational change in Hha that exposed residues deeply buried in its hydrophobic core. Conformational plasticity of Hha could be evidenced even in the absence of H-NS. A temperature shift from 25 to 37 ºC results in a dynamic equilibrium involving different Hha conformations. Interestingly, the most perturbed residues by the temperature-induced structural alteration partially overlap with those observed when Hha interacts with H-NS$_{64}$ (Garcia et al. 2005).

In contrast, H-NS amino acid residues interacting with Hha are located in helices H1 and H2 of the N-terminal domain. The relevance of this region is highlighted by the fact that in *E. coli*, point mutations consisting in replacement of Arg^{12} or Arg^{15} by either histidine or cysteine induced de-repression of the *proV* operon under low osmolarity conditions (Uegu-chi et al. 1996). We have demonstrated that Arg^{12} and Arg^{15}, located in the same side and

in adjacent turns of helix H2, are involved in different protein-protein interactions: Arg^{12} is essential for Hha binding without affecting the fold of the $H\text{-}NS_{46}$ dimer, and Arg^{15} does not affect Hha binding but is essential for proper folding of $H\text{-}NS_{46}$ dimers (Fig. 1). The $Arg^{12}His$ mutant was used to confirm that all relevant interactions with Hha occur only in the N-terminal domain of H-NS, as the introduction of this mutation in full length H-NS also abolished Hha binding (GARCIA et al. 2006).

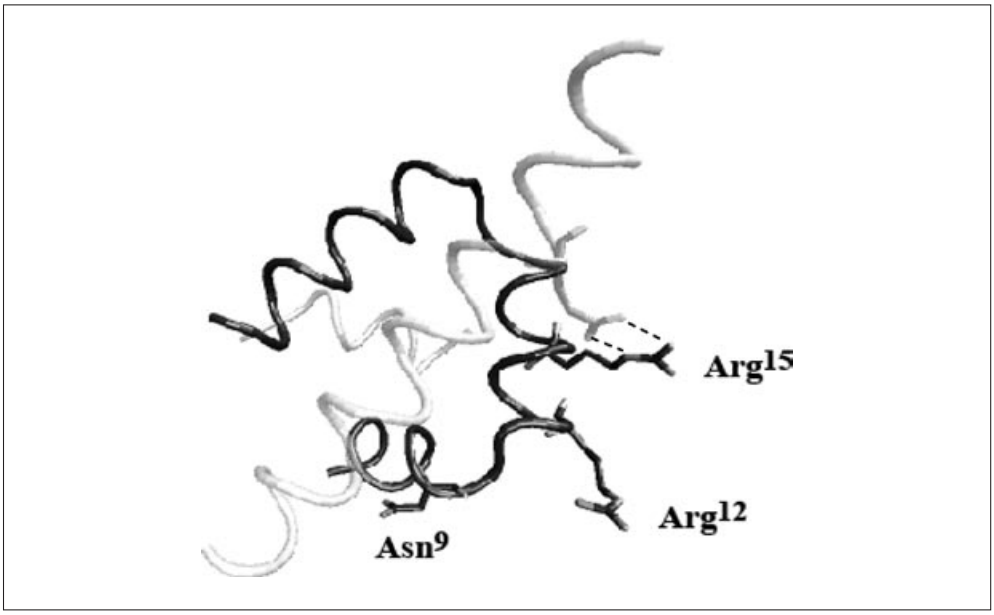

Fig. 1 Location of key H-NS residues. Expanded view of the antiparallel structure of *E. coli* $H\text{-}NS_{46}$ showing the position of key residues for Hha binding: Asn^9 and Arg^{12}. The interactions involving residue Arg^{15} and glutamic acid side chains in the second protomer are also shown. The two protomers are displayed in white and black.

A comparison of the sequences of H-NS-like proteins from Gram-negative bacteria shows that the region including residues 9 to 15 (*E. coli* H-NS numbering), involved in Hha binding, is strictly conserved in bacteria of the *Enterobacteriaceae* family that also contain Hha-like proteins in their genome. Species of the genus *Vibrio* that are phylogenetically close to the *Enterobacteriaceae*, do not encode Hha-like proteins and show a lower degree of conservation in the Hha-binding region, although Arg^{12} is completely conserved also in these species (Tab. 1).

The conservation of this residue may suggest an evolutionarily older conserved role, and the interaction of Hha with Arg^{12} may have appeared as a way of modulating this putative role. On the basis of these observations, we decided to investigate if other residues, besides Arg^{12}, are directly implicated in Hha binding. Substitutions at positions 7, 9 and 13 could be found in H-NS proteins from species of the genus *Vibrio*. Since those positions are highly conserved (positions 9 and 13 are strictly conserved) in H-NS proteins from Hha-containing bacteria, we speculated that they may be important for a specific function (Hha binding) that is not shared with other H-NS proteins. To assess the contribution of each individual

Tab. 1 Correlation between amino acid conservation in the Hha-binding region of H-NS and the presence of *hha*-like genes in the bacterial chromosome

Bacterial species	Protein	Hha-binding region	*hha* gene
Escherichia coli	H-NS	7ILNNIRTLR15	+
Sigella flexneri	H-NS	7ILNNIRTLR15	+
Salmonella enterica serovar *Typhimurium*	H-NS	7ILNNIRTLR15	+
Yersinia enterocolitica	H-NS	7ILNNIRTLR15	+
Erwinia carotovora	H-NS	7ILNNIRTLR15	+
Escherichia coli	StpA	7SLNNIRTLR15	+
Vibrio cholerae	H-NS	7TLLNIRSLR15	–

amino acid in Hha binding, one of those residues at a time was replaced by the corresponding residue found at that position in *V. cholerae*. Using NMR, we tested the *in vitro* binding capacity of those H-NS mutants to Hha. The results obtained clearly showed that residue Asn^{9} plays a key role in Hha-binding and its substitution for leucine produced a strong decrease in binding affinity. Other H-NS residues within the Hha-binding motif, such as Ile^{7} and Thr^{13}, whose NMR signals were strongly perturbed by the addition of Hha to wild type $H\text{-}NS_{46}$, could be replaced by the residues found at that position in *V. cholerae* H-NS without a significant effect in Hha binding.

2. Conclusions

Using a combination of mutagenesis and NMR we have studied the interaction between the *E. coli* H-NS and Hha proteins. Our results indicate that a conformational change in Hha is involved in the interaction. With respect to the H-NS protein, the Hha binding motif was identified within helices H1 and H2 of the H-NS N-terminal domain, where Asn^{9} and Arg^{12} side chains may be required for polar interactions at the center of the H-NS/Hha interface.

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Der Knochen als Archiv

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Als hochaktives Organ des Stoffwechsels und zugleich kompaktes, lange Zeiträume überdauerndes Material ist der Knochen in vielfältiger Weise Träger unterschiedlichster Informationen. Einerseits wurden Knochen – sei es von Tieren oder vom Menschen – von frühen Kulturen bis heute immer wieder als Werkstücke für die Herstellung von kunsthandwerklichen Schriftträgern und Objekten benutzt, andererseits spiegeln Struktur und Architektur der Knochenmatrix sowie ihr molekularer Aufbau getreu die Einflüsse der täglichen Umwelt wider und lassen uns einen Blick zurück in die Umweltsituation vergangener Epochen werfen. Das hohe Maß an Reaktionsfähigkeit des gesunden wie des kranken Knochengewebes geben uns Einblicke in die individuelle Geschichte der Beanspruchung des einzelnen Knochens und lassen uns die Mechanismen der Evolution des Skeletts besser verstehen.

Die Beiträge spannen daher den Bogen von den Fossilarchiven der Hominisation, über Tierknochen als Archive für Klima und Landschaft, aber auch als molekulares Archiv, genetische Komponenten ihrer Struktur, Geo- und Bioelemente in Knochen, den Werkstoff Knochen, den Knochenersatz und die Determinanten mechanischer Kompetenz der Knochen bis hin zur forensischen Bedeutung und der Darstellung von Knochen in der Grabdenkmalskunst.

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The H-NS Protein from *Escherichia coli* and Related Enterobacteria: a Bacterial Molecular Neural Network

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With 1 Figure

Abstract

A relevant feature of the H-NS protein encoded by members of the family *Enterobacteriaceae* is its ability to interact with proteins that structurally mimic its N-terminal domain. This interaction results in fine tuning of the H-NS modulatory capability. The all-helical structure of one of these proteins, the Hha protein, appears to be conserved in other proteins that play a role as global modulators of gene expression (e. g., YmgB protein).

Zusammenfassung

Ein wichtiges Merkmal des von Mitgliedern der Familie der Enterobacteriaceen gebildeten H-NS-Proteins ist dessen Fähigkeit zur Interaktion mit Proteinen, deren Struktur der N-terminalen Domäne des H-NS ähneln. Diese Interaktion führt zur Feinabstimmung der modulatorischen Eigenschaften des H-NS Proteins. Die rein helikale Struktur eines dieser Proteine, des sogenannten Hha-Proteins, scheint auch in anderen Proteinen, die als globale Modulatoren der Genexpression eine Rolle spielen, konserviert zu sein.

1. State of the Art

The nucleoid-associated proteins (NAPs) constitute a superfamily of proteins that have genome structuring functions in bacteria. The association of many of these proteins with DNA influences not only its conformation, but also DNA replication, recombination and transcription. Best characterized in *Escherichia coli* and related genera of the *Enterobacteriaceae*, H-NS is a good example of a NAP with functional dichotomy: it contributes to bacterial chromosome architecture and can generate nucleoprotein complexes in the vicinity of specific promoters to influence transcription. The H-NS protein contains two well-defined domains connected by a flexible linker. The C-terminal domain includes se-

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quences absolutely required for DNA binding, whereas the N-terminal domain (oligomerization domain) allows the protein to generate homo- and hetero-oligomers. Generation of homodimers and oligomers is underlying H-NS-mediated repression of transcription. In addition to homomeric interactions H-NS is also capable of heteromeric interactions. Whereas some heteromeric interactions also influence the repressory ability of H-NS, other interactions influence cellular processes different from transcription, such as transposition.

Proteins interacting with H-NS may contain protein- and DNA-binding domains, as its paralogue StpA, or just protein-binding domains, as the members of the Hha/YmoA family of proteins. Proteins of this latter family show structural mimicry to the H-NS oligomerization domain and co-regulate with H-NS several virulence determinants in enteric bacteria (Madrid et al. 2007a). The Hha-YmoA family of proteins includes a group of sequence-related low molecular mass proteins (molecular mass of about 8 kDa) involved in gene regulation that are encoded by *E. coli* and related members of the *Enterobacteriaceae*. Genes coding for such proteins can be present in one or more copies per chromosome or in conjugative plasmids. H-NS binding to any of these proteins results in an increased repressory ability of the complex. On the other hand, the ability of H-NS to repress transcription can also be reduced by interacting with a different type of truncated H-NS-like protein: the $H\text{-}NST_{EPEC}$ protein expressed by some enteropathogenic *E. coli* strains (Williamson and Free 2005). Fine-tuning of H-NS modulatory ability is therefore accomplished by heteromeric interactions with proteins that show structural mimicry to its oligomerization domain. Protein-protein interaction appears as a relevant feature of the ability of H-NS to sense environmental stimuli and modulate different cellular processes. Genomes encoding for both *hha* and *hns* genes correspond exclusively to members of the family *Enterobacteriaceae*, including some of the endocellular obligate symbionts that belong to the family (*Wigglesworthia glossinidia* and *Sodalis glossinidius*) (Madrid et al. 2007b).

Structural details of Hha-H-NS interaction are available. With respect to the Hha protein, amino acid residues interacting with H-NS are scattered along the whole molecule (Fig. 1). In contrast, H-NS amino acid residues interacting with Hha are located mainly within helixes H1 and H2 of the H-NS N-terminal domain (García et al. 2005). If the sequence corresponding to the N-terminal end of the H-NS protein is used to perform an alignment, a seven amino acid sequence (LNNIRTL) located within helixes H1 and H2 is absolutely conserved among the H-NS proteins encoded by those microorganisms that encode a chromosomal *hha*-like gene. That seven-amino acid stretch is included within the H-NS domain that interacts with Hha, and corresponds with the residues most affected after interaction with Hha (Madrid et al. 2007b). It has been proposed for that sequence the term Hha signature. With the genomic data available, the intact Hha signature of the H-NS protein is exclusively found in chromosomally-encoded H-NS proteins from members of the family *Enterobacteriaceae*.

The three-dimensional structure of the Hha protein consists of four alpha-helical segments separated by loops: helix 1 (residues 8–16), helix 2 (residues 21–34), helix 3 (residues 37–55) and helix 4 (residues 65–69) (Yee et al. 2002). Helices 1 and 2 are packed against the N-terminal half of helix 3. Helix 4 is very short and interacts with the C-terminal part of helix 3. Studies of the interaction of Hha with $H\text{-}NS_{64}$ (H-NS residues 1 to 64) showed that strongly perturbed residues are located both in the surface and in the hydropho-

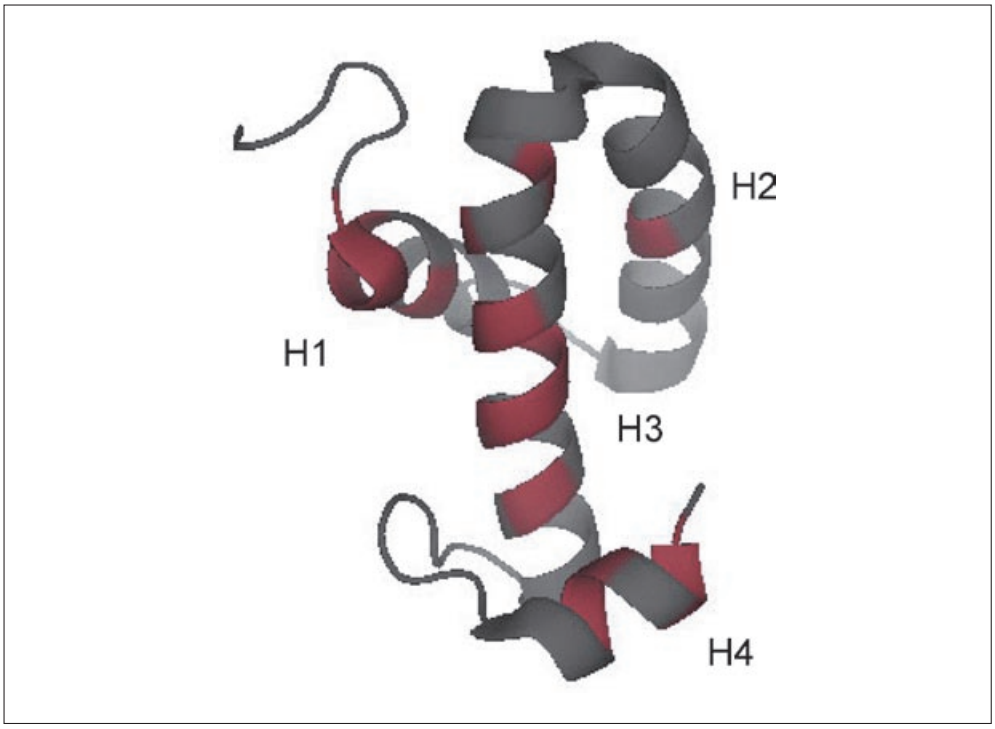

Fig. 1 Ribbon representation of three dimensional Hha structure. Residues most affected by the interaction with the H-NS protein are coloured in deep red.

bic core of the Hha structure (Fig. 1), suggesting that Hha undergoes conformational rearrangement after interacting with H-NS_{64} (García et al. 2005).

H-NS multimerization and H-NS-dependent DNA bridging are mediated by H-NS dimers and tetramers. In addition to the N-terminal domain of H-NS, the linker domain appears to be required for tetramerization and oligomerization (Stella et al. 2005). Therefore, binding of H-NS to proteins lacking the linker domain (i. e. H-NST_{EPEC}) would reduce the ability of H-NS to oligomerize and bridge DNA. This could explain the ability of the H-NST_{EPEC} proteins to interfere with the repression by H-NS (Williamson and Free 2005). On the other hand, binding of H-NS to Hha-like proteins enhances repression by H-NS, which would potentiate rather than inhibit dimerization and oligomerization. Although a precise picture of the complexes formed *in vivo* is not available, we speculate that Hha-like proteins, when interacting with H-NS, affect the oligomerization of H-NS by providing additional sites for interaction or by influencing the folding of the linker domain.

A recent finding shows that the all-helical structure of Hha is not unique within the *Enterobacteriaceae*: the three-dimensional structure of the YmgB protein, that shows only 5 % amino acid identity with Hha, is very similar to that of Hha. YmgB protein plays a relevant role in biofilm formation and acid resistance in *E. coli* and related enteric bacteria (Lee et al. 2007).

2. Conclusions

A group of low-molecular mass proteins showing an all-helical structure interact with H-NS to modulate, among other cellular processes, gene expression. H-NS interaction with these proteins is an evolutive trait of *E. coli* and related members of the *Enterobacteriaceae*.

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Virulence Factors

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Metabolic Traits Associated with Virulence of Pathogenic *E. coli* Strains

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Abstract

Colonization of the intestine is the first step of host infectivity for all pathogenic *E. coli* strains, whatever the category. Various intestinal pathogenic strains are rarely encountered in the fecal flora of healthy hosts. In contrast, extraintestinal pathogenic *E. coli* stably colonizes the intestine without inducing clinical symptoms and is the predominant *E. coli* in approximately 20 % of healthy people. Little is known about what genes and factors in pathogenic strains help them to outcompete with the normal flora and colonize the intestine. Comparative genomic analyses of pathogenic and commensal *E. coli* indicated that many DNA islands specific to pathogenic isolates harbor genes encoding proteins with metabolic functions, including aromatic degradation and transport and utilization of carbohydrates. However, the relationship between virulence and the functionality of these regions is largely unknown. Because carbohydrate metabolism is assumed to be the nutritional basis for colonization of the intestine by *E. coli* and maintenance of the strains (Chang et al. 2004), our goal is to clarify the role of sugars catabolized specifically by pathogenic *E. coli* in colonization of the intestine. We firstly studied the *deoK* operon enabling the strains to use deoxyribose as a carbon source. We initiated this study because deoxyribose is a sugar derived from DNA degradation and as both shed colonic epithelial cells and microflora are sources of DNA, the large intestine is rich in DNA. Although ability of various *E. coli* strains to degrade deoxyribose was reported a long time ago, the genes encoding this function were not identified at the beginning of our study.

Zusammenfassung

Die Besiedelung des Darmes ist bei allen pathogenen *E. coli*-Stämmen der erste wichtige Schritt für ihre Infektiosität. Verschiedene intestinale pathogene Stämme werden selten in der Stuhlflora von gesunden Wirtsorganismen gefunden. Extra-intestinale pathogene *E. coli* dagegen besiedeln den Darm, ohne dabei klinische Symptome auszulösen, und stellen die wichtigste Gruppe in etwa 20 % der gesunden Bevölkerung dar. Bisher ist wenig darüber bekannt, welche Gene und Faktoren pathogene Stämme besitzen, die es ihnen ermöglichen die normale Darmflora zu überwinden und den Darm zu besiedeln. Vergleichende Genomanalysen pathogener und kommensaler *E. coli* weisen darauf hin, dass viele, für pathogene Isolate spezifische DNA-Inseln Gene beinhalten, die für Proteine mit metabolischen Eigenschaften kodieren. Dazu gehören der Abbau aromatischer Verbindungen und der Transport und die Verwertung von Kohlenhydraten. Die Beziehung zwischen Virulenz und der Funktionalität dieser Regionen ist jedoch noch gänzlich unklar. Da man davon ausgeht, dass der Kohlenhydratstoffwechsel die Nährstoffgrundlage für die Besiedelung des Darmes durch intestinale *E. coli* und deren Versorgung dort sein könnte (Chang et al. 2004), ist es unser Ziel, die Rolle der Zucker aufzuklären, die während der Ansiedelung des Darmes von pathogenen intestinalen *E. coli* gezielt verwertet werden.

1. State of the Art

The deoxyribokinase that catalyses the ATP-dependent phosphorylation of 2-D-deoxyribose to 2-D-deoxyribose-5-phosphate, has been identified in *Lactobacillus plantarum, Selenomonas ruminantium,* and *Salmonella enterica*. In *S. enterica*, four genes (*deoK* operon), encoding a putative permease (DeoP), a deoxyribokinase (DeoK), a mutarotase (DeoM) and a transcriptional regulator (DeoQ), are involved in the use of deoxyribose (Tourneux et al. 2000, Assairi et al. 2004). We recently reported the identification and characterization of a 61-kb pathogenicity island (PAI I_{AL862}) in the AL862 sepsis isolate (Lalioui et al. 2001). Analysis of the nucleotide sequence of PAI I_{AL862} showed that it carries a *deoK* operon which is highly similar (78 % of identity) to that from *S. enterica*. Investigation of the wild type strain, mutants and transcomplemented mutants showed that the *deoK* operon is expressed in *E. coli* and that its presence correlated with the ability to use deoxyribose as a sole carbon source. We investigated pathogenic and commensal isolates from various collections for both the presence of the *deoK* operon and use of the sugar. The presence of the operon was always correlated with the use of deoxyribose. Although we found that both pathogenic and commensal strains harbored the *deoK* operon, our results strongly suggested that this operon is associated with the pathogenicity of the strain (45.4 % of pathogenic strains versus 22.9 % of commensal strains harboured *deoK*; $P < 0.01$) (Bernier-Febreau et al. 2004). In extraintestinal pathogenic isolates the frequency of deoxyribose clones significantly increased with the number of virulence factors (manuscript in preparation). Sequencing and comparison of the *deoK* operons in three pathogenic and one commensal isolates showed that this operon is conserved (98 % identity) in all the strains. A few base pairs (242 bp upstream and 115 bp downstream) directly flanking the operon corresponded to partial sequences of the *ilvN* and *uhpA* genes, between which the *deoK* operon is inserted in *S. enterica*. The high level of DNA identity, the colinearity, the similar transcriptional regulation of the genes as well as the similarity of the boundaries flanking the *deoM* and *deoQ* genes, are evidences for horizontal gene transfer of the *deoK* operon from *S. enterica* to *E. coli* (Bernier-Febreau et al. 2004). To study the advantage conferred by the expression of the *deoK* operon, we carried out co-cultures experiments. When the growth medium contains the sugar we observed that expression of the deoxyribokinase conferred to the strain a late stationary growth advantage. Finally, we investigated the role of deoxyribose catabolism in the colonization of the intestine with the 55 989 clinical isolate. We knocked out the pathway enabling deoxyribose metabolism. Then, we tested the mutant for its relative fitness for intestinal colonization in competition with its parental strain in an animal model. The 55 989 isolate outcompeted its *deoK* mutant over a long period of time under *in vivo* conditions, strongly suggesting that *deoK* operon expression plays a role in the persistence in the intestine (manuscript in preparation).

2. Conclusions

This is the first example of a sugar catabolism involved in host colonization by pathogenic *E. coli* isolates. Our study shows that deoxyribose metabolism is a biochemical characteristic transferred horizontally from *S. enterica* to *E. coli* and widely distributed among pathogenic *E. coli* isolates from various origins. Our findings suggest that acquisition of this trait

may be an evolutionary step enabling these pathogens to colonize and maintain themselves in mammalian intestine, their reservoir.

Comparative genomic and metabolic analyses allowed us to identify other metabolic traits associated with pathogenic isolates that are under investigation. Preliminary data indicate that fermentation by pathogenic *E. coli* of various sugars considered as limiting nutrients in the gut, may provide a nutritional basis for co-colonization of the intestine with the commensal *E. coli* flora.

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Life Strategies of Microorganisms in the Environment and in Host Organisms

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Viele Prokaryoten (Bakterien wie Archaeen) in Wasser, Boden oder Wirtsorganismus zeigen häufig spezifische, hoch entwickelte Anpassungen zur Nutzung des umgebenden Milieus als Energie- und Nährstoffquelle sowie als Überlebensraum. Bei prokaryotischen Organismen spielen sich die Anpassungen an die abiotische oder biotische Umgebung fast nur auf der Ebene des Stoffwechsels und der molekularen bzw. makromolekularen Strukturen und Wechselwirkungen ab. Auf dieser Ebene zeigen sich tatsächlich typisch prokaryotische Leistungen: eine Nutzung „ungewöhnlicher" Energiequellen, grundlegende Syntheseleistungen, enzymatische Katalysen, Anpassungen an extreme Verhältnisse, wie hohe Temperaturen, Kolonisations- und Invasionsmechanismen, und Regulationsvorgänge. Der Band behandelt die Rolle von Mikroorganismen in den globalen Elementkreisläufen, wie z. B. dem Schwefelstoffwechsel, dem Methanstoffwechsel, der Kohlendioxidfixierung oder der Ammoniakoxidation, Symbiosen mit Bakterien-Eukaryoten-Interaktionen auf der Grundlage von Schwefel- oder Stickstoffstoffwechsel, Modelle zum prokaryotischen Ursprung von Organellen in Eukaryoten sowie die Beteiligung von Mikroorganismen an Infektions- und Pathogenitätsvorgängen.

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The High-Pathogenicity Island of Extraintestinal Pathogenic *Escherichia coli* (ExPEC) – a Virulence Determinant Aquired by Horizontal Transfer

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Abstract

The high-pathogenicity island (HPI) is a genomic island encoding a siderophore-mediated iron uptake system, which is essential for pathogenicity of extraintestinal pathogenic *Escherichia coli* (ExPEC). The HPI is widely disseminated among *Enterobacteriaceae* which poses questions regarding its' horizontal transfer. A unique type of the HPI has been characterized in *E. coli* strain ECOR31 carrying a functional conjugative mating pair formation (Mpf) and a DNA processing system characteristic of integrative and conjugative elements (ICE). We further determined the role of the HPI and the IroN siderophore system in ExPEC-pathogenicity revealing that the IroN-receptor, but not the HPI contributes to invasion of urothelial cells by ExPECs.

Zusammenfassung

Die „High-Pathogenicity Island" (HPI) ist eine genomische Insel, die ein Siderophor-abhängiges Eisenaufnahmesystem kodiert und für die Pathogenität extraintestinaler *E. coli* mitverantwortlich ist. Auch unter anderen *Enterobacteriaceae* ist die HPI weit verbreitet, was auf einen effizienten horizontalen HPI-Transfer hindeutet. Ein spezieller Typ der HPI wurde bei dem *E. coli*-Isolat ECOR31 nachgewiesen. Diese HPI kodiert ein konjugatives Pilus- (Mpf) sowie eine DNA-Prozessierungssystem und stellt ein integratives konjugatives Element (ICE) dar. Zudem wurde die Bedeutung der HPI und des IroN-Siderophorrezeptors für die ExPEC-Pathogenität untersucht: Der IroN-Rezeptor, nicht aber HPI-Produkte sind an der Urothelzell-Invasion durch ExPEC beteiligt.

High-virulent Yersiniae (*Y. pestis*, *Y. enterocolitica* IB, *Y. pseudotuberculosis*) carry a 45–100 kb instable genomic island that is a prerequisite for virulence expression in these species. This High-Pathogenicity Island (HPI) encodes a siderophore-mediated iron-uptake system (yersiniabactin, Ybt), which is the only siderophore found in pathogenic *Yersinia* species (de Almeida et al. 1993). In contrast to the majority of pathogenicity islands described so far, the HPI reveals a unique property, as orthologous HPIs are widely distributed among different members of the *Enterobacteriaceae* (Schubert et al. 2000). These homologous HPI are distinctively distributed among clinical isolates of extraintestinal pathogenic *E. coli* (ExPEC) and of other members of the family *Enterobacteriaceae* isolated from blood cultures, urine samples and cerebrospinal fluid (CSF) (Schubert et al. 1998). Moreover, the functional HPI contributes to extraintestinal virulence of ExPECs as has been shown in mouse models of urinary tract infection and peritonitis (Schubert et al. 2002). The nucleotide sequence identity between the HPI of *E. coli* and *Yersinia pestis* is higher

than 99 % indicating a very recent transfer of the HPI between these species. The findings of such a promiscuous transfer of the HPI among different genera of the family *Enterobacteriaceae* poses interesting questions regarding the mechanism of HPI transfer. The first step of horizontal transfer of the HPI requires a mobilization of the chromosomal island generating a transferable genetic element, which closely resembles the initial step of transfer of lambdoid phages. Enzymes such as a P4-like integrase and an excisionase together with distinct DNA structures, the attachment sites *attR* and *attL*, are required for the mobilization process. In contrast to the *Yersinia*-HPI, which is flanked by typical attachment sites (*attL* and *attR*) involved in integration and excision of the island, almost all *E. coli* strains investigated to date revealed a deletion within the 3′-border of the HPI leading to a loss of the attachment site *attR*. Thus, the majority of HPIs in ExPEC appear to be fixed in the host chromosome. As our first approach in characterizing the HPI of *E. coli* (HPI_{Ec}) revealed exclusively HPIs with right-handed truncations, we performed a systematic survey of the presence and location of the HPI by analyzing the well-defined *E. coli* collection of reference (ECOR) strains. Surprisingly, we identified the HPI of a certain *E. coli* strain of this collection, ECOR31, (HPI_{ECOR31}) to be inserted at the *asnV* t-RNA gene. This HPI_{ECOR31} further carries a 35-kb DNA region downstream of the *fyuA* gene, which is located exactly at the position where an IS*100* element is inserted into the HPI of *Y. pestis*/*Y. pseudotuberculosis*, and includes an intact *attR* site (Schubert et al. 2004). The structure and the mobilization of the HPI_{ECOR31} are reminiscent of both temperate bacteriophages and conjugative plasmids. The generation of an extrachromosomal intermediate is likely to be the essential step in the successful transfer of the HPI_{ECOR31} and must precede conjugative transfer to recipient cells.

This finding may provide a missing link in the evolution of the HPI in general as it encompasses three distinct regions encoding:

(*i*) a complete and functional mating pair formation (Mpf) system related to IncX plasmid R6K of *E. coli*,
(*ii*) a putative *nic* site (origin of transfer, *oriT*) together with a DNA-processing region related to plasmid CloDF13 of *Enterobacter cloacae* (MobB, MobC), and
(*iii*) ORFs displaying a weak homology to chromosomal genes of *Vibrio cholerae*.

We could demonstrate, that the *nic* site is recognized by the specific nicase (MobB/C) and that both Mpf system and DNA-processing region are required for the conjugative transfer of a subcloned HPI_{ECOR31} (Schubert et al. 2004). Together with the unique structure of the HPI_{ECOR31} encoding a DNA-processing system, pilus assembly and mating pair formation functions typical for conjugative DNA transfers, the entire HPI_{ECOR31} structurally resembles a subgroup of integrative and conjugative elements (ICE) (Burrus et al. 2002, Hochhut and Waldor 1999) and is suggestive of being a mobilizable progenitor of the HPI found in both *E. coli* and *Y. pseudotuberculosis*/*Y. pestis*.

The production of yersiniabactin siderophore encoded by the HPI is remarkable as ExPEC strains possess more than four different high efficient iron uptake systems, e. g. is the enterobactin-, aerobactin- and salmochelin system. The contribution of these various siderophore uptake systems for the *E. coli* life cycle is yet unknown. However, it has been shown in animal models that the gene expression of these iron-uptake systems is up-regulated during infection. The functional redundancy of five and more iron uptake systems is a unique feature of ExPECs, which may indicate that siderophore receptors fulfill additional tasks

in the pathogenicity of ExPECs. In this regard, the recently identified siderophore receptor IreA (iron-responsive element) has been demonstrated to function both, as siderophore receptor and as an adhesin in UTIs (Russo et al. 2001). A recent study characterized the outer membrane protein Iha as both catecholate siderophore receptor and adherence factor of ExPEC strains (Leveille et al. 2006). Previous epidemiological studies revealed a high prevalence of FyuA and IroN in ExPEC strains (Clermont et al. 2000, Duriez et al. 2001, Johnson et al. 2005). There is also accumulating experimental evidence that the IroN protein is involved in the infection process of ExPECs (Negre et al. 2004, Russo et al. 2002, 2003). However, these studies failed to provide evidence about the direct involvement of IroN in invasion of urothelial cells. We could demonstrate for the first time that the catecholate receptor IroN contributes to the invasion of urothelial cells by ExPECs. The findings corroborate the hypothesis that siderophore receptors may exert a dual role in the pathogenesis of ExPEC, as they facilitate both metabolic functions and host cell interaction. We postulate a putative host receptor responsible for the IroN-mediated invasion and suggest a mechanism by which IroN contributes to urovirulence. Taken together, these results demonstrate that the salmochelin receptor IroN contributes to the invasion of ExPEC strains *in vitro* (Feldmann et al. 2007). In contrast, the yersiniabactin receptor FyuA encoded by the HPI did not appear to have any direct affect on the invasion of ExPEC strains *in vitro*. No increase of adherence was conferred by the presence of IroN suggesting that IroN-mediated invasion is not due to enhanced adherence. Instead, IroN may directly trigger uptake of ExPEC into host cells. Furthermore, we could observe that the IroN-mediated invasion is not unique to HCV29 cells, as similar invasion results were obtained using different urothelial cell lines.

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Genetic Diversity of Extraintestinal Pathogenic *Escherichia coli* Isolated from 161 Patients with Bacteremia

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Abstract

Extraintestinal pathogenic *E. coli* (ExPEC) are important causes of bacteremia and sepsis worldwide. Paradoxically *E. coli* is present in the feces of every human being; therefore being able to identify the strain which can disseminate may help to prevent one of the major causes of sepsis. In order to achieve this goal, we studied the genetic diversity of 161 consecutive *E. coli* strains isolated from blood culture during one year in two major University Hospitals in Paris. Clinical data were collected. All isolates were characterized by multilocus sequence typing (MLST), and for the presence of nine virulence factors (VFs). Sequence-based phylogenetic analysis revealed a strong concordance with the four classical phylogenetic groups (A, B1, B2 and D). Profile-based analysis of MLST data distinguished 87 sequence types, which were grouped into 19 clonal complexes (CCs). No CC was particularly associated with a severe sepsis or death of the patient. However, when compared with the previously reported genetic diversity of other sets of *E. coli* strains obtained from various origins, our data do not support the hypothesis that the ability to be invasive is restricted to a small subset of strains.

Zusammenfassung

Extraintestinale pathogene *E. coli* (ExPEC) sind weltweit eine wichtige Ursache für Bakteriämie- und Sepsiserkrankungen. Paradoxerweise kommen *E. coli* im Fäzes jedes menschlichen Wesens vor. Wenn es möglich wäre, diejenigen Stämme, die sich ausbreiten können, zu identifizieren, könnte dies dazu beitragen, eine der Hauptursachen für Sepsis auszuschalten. Um dieses Ziel zu erreichen, haben wir die genetische Diversität von 161 *E. coli*-Stämmen untersucht, die innerhalb eines Jahres aus Blutkulturen zweier Universitätskliniken in Paris isoliert wurden. Klinische Daten wurden ebenfalls gesammelt. Alle Isolate wurden mit Hilfe des „Multilocus Sequence Typing" (MLST) charakterisiert und hinsichtlich des Vorkommens von neun verschiedenen Virulenzfaktoren (VF) untersucht. Die Sequenz-basierte phylogentische Analyse zeigte eine starke Übereinstimmung mit den vier klassischen phylogenetischen Gruppen A, B1, B2 und D. Mit Hilfe der MLST-Analyse konnten 87 Sequenztypen unterschieden werden, die in 19 klonale Komplexe (CCs) gruppiert wurden. Kein CC war mit einer schweren Sepsis oder dem Tod eines Patienten assoziiert. Vergleicht man jedoch unsere Daten mit kürzlich veröffentlichten Datenreihen zur genetischen Diversität von *E. coli*-Stämmen verschiedenen Ursprungs, so unterstützen sie nicht die Hypothese, dass die Fähigkeit zur Invasivität nur auf einen kleinen Teil der Stämme beschränkt ist.

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Escherichia coli is the most abundant aerobic bacteria of the human intestinal flora. Whereas *E. coli* usually appears to be a harmless commensal, in some circumstances, it becomes pathogenic (Dobrindt 2005). Among the various pathotypes of *E. coli* strains, the most common one is due to extraintestinal pathogenic *Escherichia coli* (ExPEC) which are responsible for urinary tract infections, intra-abdominal and soft tissue infections, meningitis, pneumonia and osteomyelitis often leading to bacteremia (Russo and Johnson 2000). Some VFs, such as adhesins, capsule, cytotoxins and siderophores, are necessary to overcome host defenses, invade host tissues and trigger a local inflammatory response (Johnson et al. 2003). Phylogenetic analyses (Selander et al. 1987, Herzer et al. 1990) suggested that *E. coli* can be divided into four main groups (A, B1, B2 and D). ExPEC have been shown to belong mostly to group B2 and, to a lesser extent, group D (Johnson and Stell 2000, Russo and Johnson 2000, Johnson and Russo 2002).

To assess whether a link could be established between a phylogenetic group and the outcome of infection, we performed a 1-year prospective cohort study of all episodes of *Escherichia coli* bacteremia in two French university hospitals. The influence of host and bacterial determinants on the initial severity and outcome of *E. coli* sepsis was assessed. Clinical data (community-acquired/nosocomial infection, immune status, underlying disease, primary source of infection, severity sepsis scoring and outcome), phylogenetic groups (A, B1, D and B2), nine virulence factors (VFs) (*papC*, *papGII*, *papGIII*, *sfa/foc*, *hlyC*, *cnf1*, *iucC*, *fyuA* and *iroN*) and the antibiotic susceptibility of isolates were investigated. All VFs except *iucC* were significantly more prevalent ($p < 0.05$) among the B2 group isolates. The non-B2 isolates were more frequently resistant to antibiotics than were B2 isolates ($p < 0.05$). There were significantly more B2 isolates from immunocompetent than from immunocompromised patients ($p < 0.05$). No bacterial or host determinants influenced the initial severity of sepsis. A factorial analysis of correspondence allowed two populations of isolates to be distinguished: those belonging to the B2 group were associated more frequently with susceptibility to antibiotics, community-acquired infection, a urinary tract origin and immunocompetent hosts; those belonging to the A, B1 or D groups were associated more frequently with resistance to antibiotics, a nosocomial origin, a non-urinary tract source and immunocompromised hosts. No influence of host or bacterial determinants on the initial severity of sepsis was detected.

The difficulty to establish a link between a phylogenetic group on one hand, and severity of infection in humans on the other hand, can have several causes. Indeed, clonal structure within phylogenetic groups and heterogeneity among clonal groups in terms of severity could constitute an important confounding factor. Characterization of sub-categories within phylogenetic groups relies on the markers that are used. Multilocus Sequence Typing (MLST) has emerged as a powerful analysis tool (Maiden et al. 1998, Enright and Spratt 1999, Urwin et al. 2003). MLST indexes the neutral genetic variation in housekeeping genes, which evolve slowly because they are under stabilizing, and not directional, selective pressure. MLST analysis *sensu stricto* consists of comparing strains based on their allelic profile, in order to minimize the disturbing effect of homologous recombination on the deduced phylogenetic relationships among closely-related strains. However, MLST data are also amenable to sequence-based phylogenetic analysis, which provides a complementary view of the population structure, and allows identifying recombination events (Feil 2004).

Several MLST studies, based on a number of distinct sets of genes, and involving either profile-based or nucleotide sequence-based analysis methods, were used to characterize

clones or phylogenetic subgroups within the *E. coli* species (Nemoy et al. 2005, Tartof et al. 2005, Goldberg et al. 2006, Johnson et al. 2006, Wirth et al. 2006, Bidet et al. 2007b, Lacher et al. 2007, Le Gall et al. 2007, Walk et al. 2007). MLST analysis of selected ExPEC strains showed that most major ExPEC clonal groups, including those characterized by antigens O47:K1, O18:K1:H7, O6:K2:H1, O6:H31 and O4, belong to phylogenetic group B2 (Johnson et al. 2001b, Bidet et al. 2007a, Le Gall et al. 2007). Other subgroups, including the multidrug resistant "clonal group A" (CGA), the O15:K2:H1 clonal group and the sepsis and meningitis associated O1:K1:H- and O7:K1:H groups, belong to phylogenetic group D (Manges et al. 2001, Johnson et al. 2002). However, the identity and distribution of ExPEC clonal groups in unselected bacteremia episodes has not been investigated systematically, and thus, the overall diversity and distribution of ExPEC strains is currently unclear. Identification of a genetic specificity linked to ExPEC is of the utmost importance in order to specifically eradicate highly invasive bacteria. Using the above described collection of strains we made an attempt at establishing an association between some clonal groups, and clinical determinants such as primary source of infection or severity of sepsis and outcome.

In this study of 161 bacteremic *E. coli* isolates, we used MLST to characterize strains. The MLST scheme is described at www.pasteur.fr/mlst. Sequence-based phylogenetic analysis revealed a strong concordance with the four classical phylogenetic groups (A, B1, B2 and D). However, we found that bacteremia isolates of group B1 were clearly associated with a urinary tract origin, which is not usually described (Johnson 1991, Picard et al. 1999, Moreno et al. 2005). The amounts of polymorphism that we found among bacteremia strains were slightly lower than a recent study of 185 isolates from freshwater beaches (Walk et al. 2007). For example, the authors found 49 *uidA* alleles with 12 % polymorphic sites, whereas we found 27 *uidA* alleles and 9.3 % polymorphic sites. Of note, our collection of bacteremic isolates included only 7 % of strains of group B1, whereas this group represented 56 % of isolates from the environment (Walk et al. 2007).

B2 subgroups were also identified by a sequence-based phylogenetic approach (Le Gall et al. 2007) using the same genes as us (except *uidA*). There was a good correlation between these B2 subgroups and our CCs, as deduced from the common strains in both studies. Our collection of bacteremic isolates of phylogenetic group B2 appears similar, in terms of number of clonal complexes or subgroups recovered, to previous collections with more diversified origins.

Our initial hypothesis was that clones (STs or CCs), rather than entire phylogenetic groups, may be more relevant natural entities to establish an association of genotype with phenotype, including clinical correlates of bacteremic isolates. Interestingly, several correlations were established between some CCs and clinical data. Most notably, two specific CCs from the B2 phylogenetic group were clearly associated with strains responsible for urosepsis. Furthermore, we found an almost complete concordance between the presence of the *svg* ORF and ST1, consistent with association of *svg* with Whittam's ST29 (Bidet et al. 2007b).

Our results confirm that most of the usually recognized extra-intestinal VFs (e. g. *pap*, *sfa*, *hly*) were concentrated in some CCs, particularly those belonging to phylogenetic group B2. In contrast, the others VFs, related to the iron uptake system (e. g. *iucC*, *iroN*, *fyuA*), were more broadly distributed (Boyd and Hartl 1998, Johnson et al. 2001a). This divergent pattern of phylogenetic distribution may reflect the location of these VFs, that are chromosomally-encoded (*pap*, *sfa*, *hly*) or plasmid-encoded (*iucC*, *iroN*, *fyuA*). On the other hand,

UPEC isolates possess specific VFs, which enable the bacteria to adhere to uroepithelial cells and to establish urinary tract infections. Most of the traditionally recognized extra-intestinal VFs (adhesins, toxins, capsules, iron uptake systems) contribute to the virulence of the strains (Johnson 1991, Russo and Johnson 2000). In this study, an association between some sets of VFs such as adhesins (*papC*, *papGII*, *sfa*), toxins (*hlyC*, *cnf1*), iron uptake systems (*iucC*, *fyuA*, *iroN*) and urosepsis was found as it was reported previously (Johnson 1991). These VFs occurring together in patterns suggested either co-selection or direct genetic linkage (Johnson and Stell 2000). Genetic linkage of VFs has been demonstrated within pathogenicity-associated islands (PAIs) and on plasmids (Hacker and Kaper 2000).

In conclusion, we characterized the clonal diversity of all consecutive isolates responsible for bacteremia during one year in two hospitals, revealing the distribution of genotypes in an unbiased way. These data clearly demonstrate the great genetic diversity of ExPEC thus suggesting that the ability to become invasive is not restricted to a small subset of *E. coli* strains. Nonetheless, some clonal complexes seem to be associated with specific clinical presentations.

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BVD – eine (un)heimliche Rinderseuche

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Die Rinderseuche BVD, die Bovine Virusdiarrhoe, mit ihrer letalen Form, der *Mucosal Disease*, ist, im Gegensatz zur BSE, der Bovinen spongiformen Enzephalopathie, keine Zoonose, sie ist für den Menschen an sich ungefährlich, aber dennoch für Rinderhalter von beträchtlicher wirtschaftlicher Bedeutung. Der BVD-Virus nutzt für seine Weiterverbreitung den noch vom Immunsystem ungeschützten fetalen Organismus als Eintrittspforte. Die Seuche lebt in den persistent infizierten Tieren weiter.
Der Band gibt einen Überblick des gegenwärtigen Forschungsstandes zu BVD. Er behandelt den BVD-Virus und die Infektion, die Erkrankung, Immunität und Immunisierung, die BVD-Erregerdiagnostik sowie die erforderlichen Maßnahmen der Tierseuchenbekämpfung.

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Genome Fluidity and its Impact on Virulence Gene Expression and Evolution of Extraintestinal Pathogenic *E. coli*

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With 2 Figures and 1 Table

Abstract

Escherichia coli is a heterogeneous species including pathogenic and commensal variants with highly dynamic genomes. Extraintestinal pathogenic *E. coli* (ExPEC) express several virulence-associated factors which are usually encoded by mobile genetic elements. Several such determinants are shared between ExPEC and certain commensal strains thus interfering with an unambiguous strain typing. Comprehensive analysis of ExPEC and commensals indicates that not only gene acquisition and DNA rearrangements, but also reductive evolution contributes to the adaptation of ExPEC to different growth conditions. Furthermore, despite of sharing virulence-associated genes with commensals, the regulation of expression of such genes may differ in ExPEC and commensals thus contributing to the establishment of extraintestinal infections.

Zusammenfassung

Escherichia coli ist eine heterogene Spezies mit hoch variablem Genom, die sowohl pathogene als auch kommensale Varianten umfasst. Extraintestinal pathogene *E. coli* sind durch das Vorkommen verschiedener Virulenz-assoziierter Faktoren charakterisiert, die oft auf mobilen genetischen Elementen kodiert vorliegen. Manche dieser Virulenz-assoziierten Gene liegen auch in kommensalen *E. coli* vor, was eine eindeutige Typisierung erschwert. Prozesse der Genomplastizität wie DNA-Aufnahme durch horizontalen Gentransfer, DNA-Umlagerungen und Genverlust tragen zur Adaptation von ExPEC an verschiedene Wachstumsbedingungen bei. Weiterhin kann es durch Genomplastizität zu einer unterschiedlichen Regulation von Genen kommen, die in ExPEC und Kommensalen vorliegen, und so ebenfalls zur Pathogenität von ExPEC beitragen.

1. *Escherichia coli* Genomes are Highly Dynamic

The species *Escherichia coli* exhibits considerable physiological and metabolic versatility and includes a variety of non-pathogenic, commensal variants, which belong to the normal gut flora of humans and many animals. Additionally, several pathogenic variants have been identified which cause various types of intestinal or extraintestinal infections in men and

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animals. Eight complete *E. coli* genome sequences are currently available, and about 40 additional complete genome sequences of pathogenic and non-pathogenic *E. coli* isolates (sequenced by different institutions) will be available in the near future. Data from comparative genomics support the hypothesis of widespread involvement of horizontal gene transfer in the evolution of *E. coli*, leading to the presence of distinct and variable "genomic islands" within the conserved "chromosomal backbone" in several bacterial lineages. Accordingly, the *E. coli* genome is composed of a conserved core of genes providing the backbone of genetic information required for essential cellular processes (Dobrindt 2005, Brzuszkiewicz et al. 2006), with an additional, flexible gene pool (Fig. 1, Tab. 1). The latter one consists of strain-specific "assortments" of genetic information, which provide additional metabolic and pathogenic properties (e. g., virulence-associated factors, antibiotic resistances) enabling these strains to adapt to special environmental conditions due to increased fitness and colonization capacity. A major constituent of the flexible gene pool is represented by accessory and mobile genetic elements and genomic or pathogenicity islands. The genomic islands are seldom fixed but rather bear the potential for ongoing rearrangements, deletions and insertions as well as for their transmission by horizontal gene transfer. Accordingly, the stable chromosomal backbone and the flexible gene pool are constantly undergoing repeated insertions and deletions (Dobrindt et al. 2004, Ahmed et al. 2008) (Fig. 1).

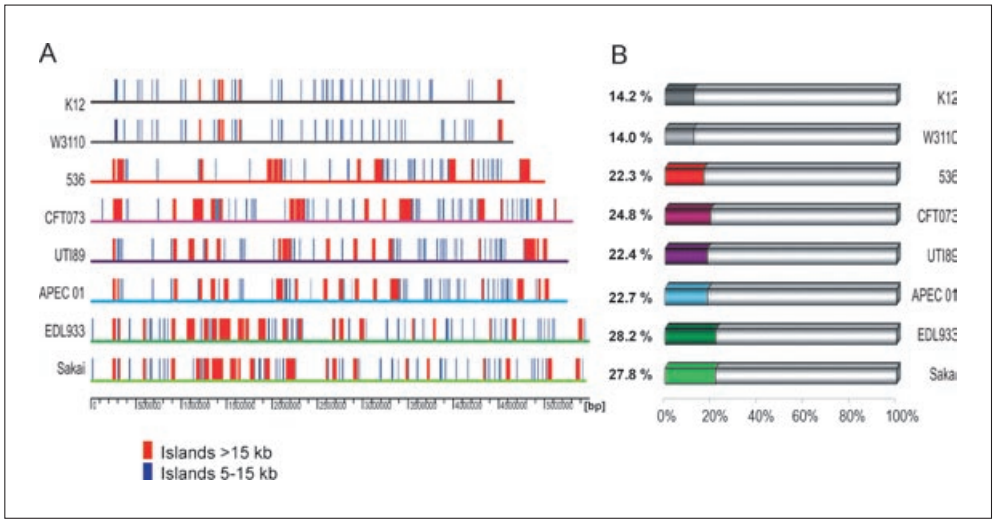

Fig. 1 Genome structure and composition of completely sequenced *E. coli* genomes. The chromosomal localization and the size of genomic islands/pathogenicity islands (blue or red bars) (*A*) as well as the overall fraction of the core- and flexible gene pool per genome (*B*) is indicated. Islands, genomic or pathogenicity islands; K12, non-pathogenic *E. coli* K-12 strain MG1655; W3110, non-pathogenic *E. coli* K-12 strain W3110; 536, uropathogenic *E. coli* strain 536 (O6:K15:H31); CFT073, uropathogenic *E. coli* strain CFT073 (O6:K2:H7); uropathogenic *E. coli* strain UTI 89 (O18:K1); APEC O1, avian pathogenic *E. coli* strain O1 (O1:K1); EDL933, enterhemorrhagic *E. coli* strain EDL933 (O157:H7); Sakai, enterhemorrhagic *E. coli* strain Sakai (O157:H7).

In addition to gene acquisition, some *E. coli* variants are also characterized by marked genome reduction. This is true for different *Shigella* species, which from the taxonomical view point, represent one particular *E. coli* pathotype as well as for enteroinvasive *E. coli*

Tab. 1 General Features of Completely Sequenced *E. coli* Genomes

Strain	MG1655 (K-12)	W3110 (K-12)	CFT073 (UPEC) (O6:K2:H1)	536 (UPEC) (O6:K15:H31)	UTI89 (UPEC) (O18:K1:H7)	APEC O1 (APEC) (O1:K1:H7)	EDL933 (EHEC) (O157:H7)	Sakai (EHEC) (O157:H7)
Chromosome size [bp]	4, 639, 221	4, 646, 332	5, 231, 428	4, 938, 875	5, 065, 741	5, 082, 025	5, 528, 445	5, 498, 450
Plasmids [size in bp]	–		–	–	pUTI89 (114, 230)	pAPEC-O1-ColBM (174, 241) pAPEC-O1-R (241, 387) pAPEC-O1-Cryp1 (105, 834) pAPEC-O1-Cryp2 (46, 870)	pO157 (92, 721)	pO157 (92, 721) pOSAK1 (3, 308)
No. of prophage-like elements	10	n. d.	5	1	n. d.	10	16	24
No. of ORFs	4,411	4,226	5,533	4,747	5,021	4,467	5,361	5,981
G+C content [%]	50.8	50	50.5	50.5	50	50.6	50.5	50.5
No. of rRNA genes	22	22	22	22	22	22	22	22
No. of tRNA genes	87	135	88	81	98	93	100	103
No. of predicted misc. RNAs	47	n. d.	51	45	n. d.	n. d.	53	13
Backbone [%]	81	n. d.	71	77	n. d.	78.3	67	n. d.
No. of strain-specific ORFs [%] [1]	406 (9)	n. d.	867 (16)	374 (8)	n. d.	201 (4.5)	1270 (24)	n. d.

[1] For comparative analysis, each ORF was searched against all ORFs of the other *E. coli* strains using the BLAST tool. Orthologous proteins were defined with an amino acid identity of >90 % over >90 % of query and reference sequence.

(EIEC). In *Shigella* spp. and EIEC genes involved in lysine decarboxylation are inactivated or deleted compared to commensal *E. coli* strains. Lysine decarboxylation produces cadaverine, an inhibitor of the *Shigella* enterotoxin. This process of "genome reduction" illustrates their adaptation to a new host environment by the loss of genes detrimental to their new life style (Maurelli et al. 1998).

In contrast, reductive evolution is also an attenuation mechanism converting virulent uropathogenic *E. coli* to asymptomatic carrier strains. Asymptomatic bacteriuria (ABU), probably the most common form of urinary tract infection (UTI), is frequently caused by *E. coli*. In ABU patients, *E. coli* establishes a carrier state, with more than 10^5 bacteria/ml of urine, but the patients do not develop symptoms. Many ABU isolates are closely related to uropathogenic *E. coli* (UPEC) strains that cause symptomatic UTI. These ABU isolates do not express many classical UPEC virulence factors, but according to genotypic analysis they possess a large number of virulence-associated genes (Zdziarski et al. 2008). Genotypic and phenotypic analysis of selected pathogenicity factors of different ABU isolates suggested that the loss of functional type 1-, F1C- and P fimbriae as well as of α-hemolysin and long LPS O-side chain expression was due to deletions or multiple point mutations, and it has been proposed that this might be essential for these *E. coli* strains to cause ABU (Klemm et al. 2006, Zdziarski et al. 2008) (Fig. 2).

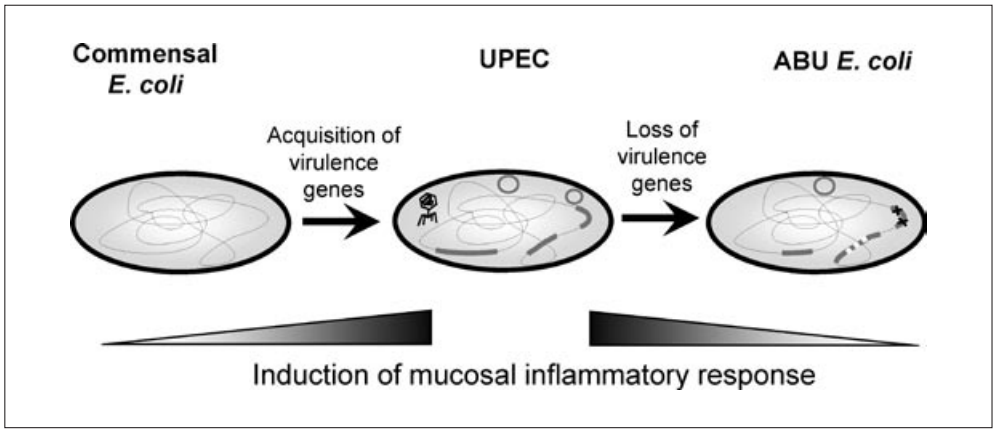

Fig. 2 Impact of genome fluidity on adaptation of uropathogenic *E. coli* and asymptomatic bacteriuria *E. coli* strains. The acquisition of mobile and accessory genetic elements such as bacteriophages, plasmids and genomic islands coding for virulence-associated genes contributes to the evolution of extraintestinal pathogenic *E. coli* from commensal variants. Genome fluidity in form of DNA rearrangements, deletions and point mutations may result in a reduced expression of such virulence associated genes or even in their inactivation or loss. The ability to down-regulate virulence factors to evade the activation of a host response may provide a competitive advantage for uropathogenic *E. coli* during infection. Over time, long-term carriage will result in the accumulation of mutations and permanent deletions in "unnecessary" virulence linked genes in asymptomatic bacteriuria isolates.

The P fimbrial determinant of these ABU isolates was always attenuated by inactivation of the *papG* gene coding for the P fimbrial adhesin due to multiple point mutations. The type 1 fimbria-encoding *fim* gene cluster was attenuated through different mechanisms: The *fim* genomic region was rather unstable with partial internal 4.2-kb deletions or larger 29-kb de-

letions including adjacent DNA stretches. Other *fim* gene clusters were inactivated by point mutations. Comparison of the *foc* determinants of different F1C fimbriae-positive and -negative ABU strains demonstrated that one particular amino acid exchange in FocD is responsible for the loss of the FocD usher activity and thus the absence of functional F1C fimbriae in these strains (Zdziarski et al. 2008). These findings exemplify the many different mechanisms of virulence attenuation that can lead to the ABU phenotype.

The loss of virulence factors is considered to reduce host response to infection and specifically, the loss of fimbriae and long chain LPS expression decreases the innate host response and bacterial clearance from the urinary tract. This suggests that the host response may drive co-evolution, and that virulence-associated genes with pro-inflammatory effects may be targeted for inactivation. In this way, ABU isolates may succeed in persisting without inducing a bactericidal inflammatory response (Bergsten et al. 2005).

The great genomic fluidity of *E. coli* results in a considerable genomic and phenotypic diversity even within clones, i. e. a group of identical or very closely related organisms: Multi locus sequence typing revealed that highly virulent UPEC, ABU and non-pathogenic *E. coli* strains can be allocated to one particular clone (Zdziarski et al. 2008). This underlines that the current MLST schemes based on conserved house keeping genes do not reliably predict the genotypes or phenotypes of individual isolates due to genome fluidity.

2. Genome Fluidity Contributes to Bacterial Adaptation due to Alterations in Gene Expression and Functional Changes

Extraintestinal pathogenic *E. coli* (ExPEC) are epidemiologically and phylogenetically distinct from a variety of commensal strains as well as from intestinal pathogenic *E. coli* (IPEC). However, in many cases ExPEC and commensal *E. coli* strains share a large fraction of their genome (Grozdanov et al. 2004). Among human ExPEC, strains can be distinguished that are involved in urinary tract infection (UTIs), sepsis, neonatal meningitis (NBM) and respiratory infection. ExPEC cause severe infections in animals as well. In poultry, *E. coli* colisepticemia is a multi-factorial disease, which involves a number of host-pathogen interactions. ExPEC and commensal strains are part of one continuum as ExPEC as facultative pathogens have the potential to become pathogenic, depending on how they accumulate and/or express virulence-associated genes. So-called "extraintestinal virulence factors" have probably evolved to enhance survival in the gut and/or transmission between hosts, and are therefore shared with at least some commensal strains. Several ExPEC virulence-associated determinants can also be found in commensal *E. coli*. Interesting examples include genes coding for microcins, fimbriae, the siderophore systems aerobactin, salmochelin and yersiniabactin, toxins (α-hemolysin, cytotoxic necrotizing factor, cytolethal distending toxin and colibactin) as well as the polyketide colibactin (Brzuszkiewicz et al. 2006, Nougayrède et al. 2006).

There is increasing evidence that ExPEC and commensal *E. coli* may also differ in the regulation of "virulence-associated" genes present in both groups of strains. The importance of ExPEC virulence-associated genes in pathogenesis appears to vary with host and tissue regarding the pathotypes, indicating the crucial role of such factors in host and tissue specificity. Moreover, the impact of these virulence factors appears to be largely depen-

dent on the specific strain background. The analysis of the presence, distribution and expression level of virulence factors which are shared by ExPEC and other *E. coli* contributes to our understanding of their evolution and role in pathogenesis. A potential zoonotic risk of human and avian ExPEC isolates cannot be excluded by the presence of specific virulence gene set and even among strains causing the same disease different alternative virulence factors can be involved in a "mix-and-match" combinatorial system fashion in each step of the infection (Mokady et al. 2005, Germon et al. 2007). Furthermore, the observation that ExPEC strains containing identical virulence-related gene signatures can be isolated from different hosts with or without disease also suggests that beside the strain genotype, the level of expression of each potential virulence-associated gene is a key factor for pathogenesis. Examples for ExPEC virulence-associated genes which are shared between different pathotypes and commensal variants but differ in their regulation are several fimbrial and other adhesin determinants.

The *agn*43 gene coding for Ag43 is widespread among the species *E. coli*, and often multiple identical or different alleles exist in the flexible genome fraction of individual isolates. Especially pathogenic *E. coli* strains frequently contain more than one similar but not always identical *agn*43 gene. The correlation between functional properties and the structure of different Ag43 variants is not yet completely understood. In addition to earlier findings that different α^{43} domain structures affect the ability to mediate autoaggregation, it becomes more and more clear that the ability to bind to components of the extracellular matrix is also dependent on the α^{43} domain structures. In this respect, genome fluidity may impact on tissue specificity and the colonization ability of *E. coli*. DNA sequence variation in the promoter regions of different *agn*43 alleles also contributes to different expression patterns of individual alleles (Beloin et al. 2006).

3. Conclusions

The *E. coli* genome is plastic and responsive to many environmental changes. Genomic alterations lead to the generation and selection of fitter mutants and thus contribute to bacterial adaptation. Host-pathogen interactions are often driven by mechanisms which involve genetic diversification, e. g. antigenic components of ExPEC are constantly under selective pressure. *E. coli* pathogens have evolved mechanisms to produce high mutation rates in specific regions of their genomes resulting in the rapid generation of variants, some of which will predominate during changing selective conditions. The analysis of ExPEC genome plasticity broadens our knowledge of pathogen evolution, adaptation and transmission dynamics of *E. coli*. *E. coli* genomic research improved our understanding of ExPEC pathogenesis in general, but as this work also impacts on the development of accurate diagnostics, surveillance and the further development of therapeutic or preventive approaches against ExPEC infections, additional efforts are required in the future to further complete our picture of *E. coli* genome plasticity.

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Reproduktionsmedizin in Klinik und Forschung: Der Status des Embryos

Leopoldina-Symposium
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Unerfüllter Kinderwunsch wird zunehmend zu einem sozialen und medizinischen Problem unserer Zeit. Seit der Geburt des ersten *in vitro* gezeugten Kindes vor fast 30 Jahren sind weltweit inzwischen mehr als drei Millionen „Retorten-Babys“ geboren worden. *In-vitro*-Fertilisation und Embryotransfer wurden zur Grundlage für die weitere Entwicklung diagnostischer und intervenierender Methoden der Reproduktionsmedizin. Mit Beginn der 1990er Jahre wandelten sich Indikations- und Methodenspektrum der assistierten Reproduktion erneut erheblich. So wurden international z. B. die intrazytoplasmatische Spermatozoeninjektion und die Präimplantationsdiagnostik möglich. Für die Reproduktionsmedizin in Klinik und Forschung besitzt der Status des Embryos eine besondere Bedeutung. Damit ist eine Vielzahl von ethischen und juristischen Fragen (z. B. Embryonenschutzgesetz, zukünftiges Fortpflanzungsmedizingesetz) verbunden. Der Band verdeutlicht das Spektrum der medizinischen Methoden und ethisch-juristischen Problemfelder und liefert sowohl Ärzten als auch ratsuchenden Patienten einen umfassenden Überblick zu den Möglichkeiten und Grenzen der modernen Reproduktionsmedizin.

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Multiepitope Vaccine against Extraintestinal Pathogenic *Escherichia coli* (ExPEC)

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With 1 Figure

Abstract

Extraintestinal pathogenic *E. coli* (ExPEC) are among the most important pathogens to cause infections in humans, such as sepsis or urinary tract infections. As ExPEC strains are becoming more and more resistant to antibiotic treatment, preventive measures such as vaccination are an achievable goal. This novel vaccine presented here is designed to target specifically ExPEC virulence factors. The vaccine was administered intranasally as well as orally using a novel antigen delivery system exploiting a bacterial Type-III secretion system. The elicited antibody titers were evaluated by ELISA essays, the cellular immune response was determined with ELISpot *in vitro* restimulation assays. Protection could be shown in a mouse model of peritonitis.

Zusammenfassung

Extraintestinal pathogene Stämme von *E. coli* (ExPEC) sind eine der häufigsten Ursachen für Infektionen beim Menschen. Sie sind u. a. für die meisten Harnwegsinfekte sowie einen Großteil der Septikämien verantwortlich. Durch steigende Resistenzraten bei ExPEC erlangen präventive Maßnahmen wie gezielte Impfungen zunehmend an Bedeutung. Der in dieser Arbeit vorgestellte neue Impfstoff ist gegen verschiedene spezifische Virulenzfaktoren von ExPEC gerichtet und wurde intranasal und über ein neues Typ-III-Sekretionssystem basiertes Impfverfahren im Mausmodell appliziert. Antikörpertiter wurden mittels ELISA, aktivierte T-Zellen mit IFN-γ-ELISpot gemessen. Die Wirksamkeit wurde im Mausmodell der abdominellen Infektion gezeigt.

1. Introduction

Extraintestinal pathogenic *Escherichia coli* (ExPEC) cause a wide variety of pathology and are responsible for a significant mortality and morbidity in humans and animals. ExPEC can be part of the normal intestinal flora, but are also able to effectively colonize and frequently infect extraintestinal sites such as the urinary tract.

The most prevalent infection mainly caused by ExPEC is cystitis. With more than 150 million cases per year cystitis is the most common bacterial infection in Europe and Northern America. About half of the women will suffer from at least one urinary tract infection (UTI) during their life time, and a significant percentage of UTI will become recurrent (Kucheria et al. 2005). Furthermore, the incidence of serious extraintestinal infection due to *E. coli* increases with age (McBean and Rajamani 2001). As the proportion of elderly patients increases in the US and other developed countries, so likely will the number

of extraintestinal *E. coli* infections. ExPECs are often also the causative agent of severe pathology such as pyelonephritis, abdominal infection, neonatal meningitis or severe sepsis. The combination of increasing numbers and increasing antimicrobial resistance predictably will make the future management of extraintestinal *E. coli* infections more challenging and costly than ever (Russo and Johnson 2003).

Thus, preventive measures such as vaccination would be an achievable goal. If preventive measures (e. g. vaccination) or therapies could be developed to specifically target the ExPEC fraction of the *E. coli* population, this could have a major beneficial impact on extraintestinal infections due to *E. coli*. For example, a vaccine strategy that leads to the development of bactericidal or interfering antibodies directed against ExPEC-specific virulence factors has the potential to prevent infections due to ExPEC without perturbing the commensal strains of *E. coli* that make up an important component of the normal intestinal flora.

Previous attempts to develop an effective ExPEC-specific vaccine, however, proved to be rather unsuccessful. Beside the ExPEC-specificity, the vaccine should consist of target structures, which are exposed to the immune system, expressed during infection and conserved within all ExPEC strains. However, the ExPEC pathotype is rather heterogeneous, and there is not a single virulence factor representative for all ExPECs. In contrast, the ExPECs exhibit a wide variety of different virulence factors such as flagella, adhesins, toxins, proteases, polysaccharide capsules and iron uptake systems (Johnson et al. 2005).

2. Vaccine Development

To construct the multi-epitope vaccine proteins, targets were selected using micro-array data and subtractive genome analysis. The target proteins are different siderophore receptors and another protein very specific for uropathogenic *E. coli*.

It has been shown that siderophore receptors specific for pathogenic *E. coli* strains are almost ideal vaccine targets, these virulence factors are expressed during infection (Snyder et al. 2004), conserved in structure and as outer membrane proteins they are partially surface exposed (Braun et al. 2000). In this study, the three dimensional structure of the proteins was simulated using several different prediction algorithms and matching them with the known crystal structure of siderophore receptor proteins FhuA and FepA. In addition, putative MHC-I and MHC-II epitopes on the target proteins were predicted with computational analysis. All epitopes were optimized for the alleles of the BALB/c mouse, the model organism used in this study. To further enhance the chance of the presentation on MHC-I receptors, the proteasome cleavage sites were also predicted and epitopes with a highly probable cleavage site on the C-terminus were preferred. Finally, all the data was manually merged in order to find short, surface exposed and epitope rich subunits of the respective target proteins. These short amino acid sequences were *in silico* combined to two recombinant proteins containing eight epitope bearing subfragments each, separated by linkers unlikely to be presented on MHC-I or MHC-II receptors. This developed amino acid sequence for two novel vaccine proteins were named Protein Vol1 and Vol2. The amino acid sequence was reverse translated into DNA using an optimized codon bias for *Enterobacteriaceae*, and excluding unwanted restriction enzyme sites, mRNA hairpin structures or internal ribosomal entry sites. Such designed genes coding for vaccine proteins 1 and 2 were synthetically generated, and the correct gene synthesis was verified by sequencing all plasmid constructs.

3. Application of the Vaccine

To evoke strong immune responses on mucosal surfaces, which are the main site of primary infection with ExPEC, mucosal vaccination strategies were used in this study. First, the vaccine proteins were expressed in *E. coli* and purified with a Histidin-tag based system and extra gel filtration. The purified protein was administered nasally with cholera toxin as an adjuvant using a booster scheme with application on days 0, 3, 7, 10 and 24.

Second the vaccine proteins were used in a bacterial antigen delivery system based on the Type-III secretion system (T3SS) of *Salmonella enterica* Serotype Typhimurium strain SB824.

The genes coding for the novel vaccine proteins were cloned as fusion products with the first 414 base pairs of the *yopE* gene of *Yersinia enterocolitica*. So the resulting protein bears the first 138 amino acids of YopE which targets the fusion protein to the T3SS of *Salmonella* (Russmann 2003) and leads to subsequent translocation into the cytoplasm of mammalian cells whenever the bacterial host strain utilizes its T3SS. The function of this system was checked in the background of the *S. enterica* SB824 strain vaccine strain, a *sptP* and *aroA* double mutant, with western blot analysis and fluorescence microscopy as shown in Figure 1.

After oral application of a solution of 5×10^8 CFU of the vaccine strains per mouse the colonization in the spleen and caecum was also determined as well as the stability of the plasmid coding for the YopE-vaccine fusion proteins. On days 25, 26 and 27 after oral vaccination the remaining Salmonellae were cleared with subcutaneous injections of ciprofloxacin.

4. Immune Response

The elicited immune response was investigated 30 days after immunization in both the intranasally and the orally vaccinated group. The increases of IgG and IgA antibody titers against the vaccine proteins were determined by ELISAs using serial dilutions of serum or vaginal wash, respectively.

The intranasally immunized mice showed a strong titer increase in IgG of serum as well as IgA of vaginal wash. The group of T3SS-vaccinated mice, however, revealed no detectable titer increase at all. To determine the amount of T-cell activation, the spleens of mice were aseptically removed 30 days post vaccination. The spleen cells were isolated by homogenization and *in vitro* restimulated with vaccine protein 1 or 2, respectively. Subsequently, IFN-γ-secreting spleen cells were detected by ELISpot indicating a highly significant increase of IFN-γ-secreting cells in the immunized groups compared to controls. This effect on cellular immune response was observed in both mice vaccinated by the T3SS-method as well as by the intranasal route.

5. Challenge in Mouse Model of Peritonitis

At day 30, the immunized and control groups of mice were challenged by intraperitoneal injection with *E. coli* CFT073. The bacterial burden of the liver was determined 24 h after challenge. Mice intranasally immunized with vaccine protein 1 and 2 showed a significant

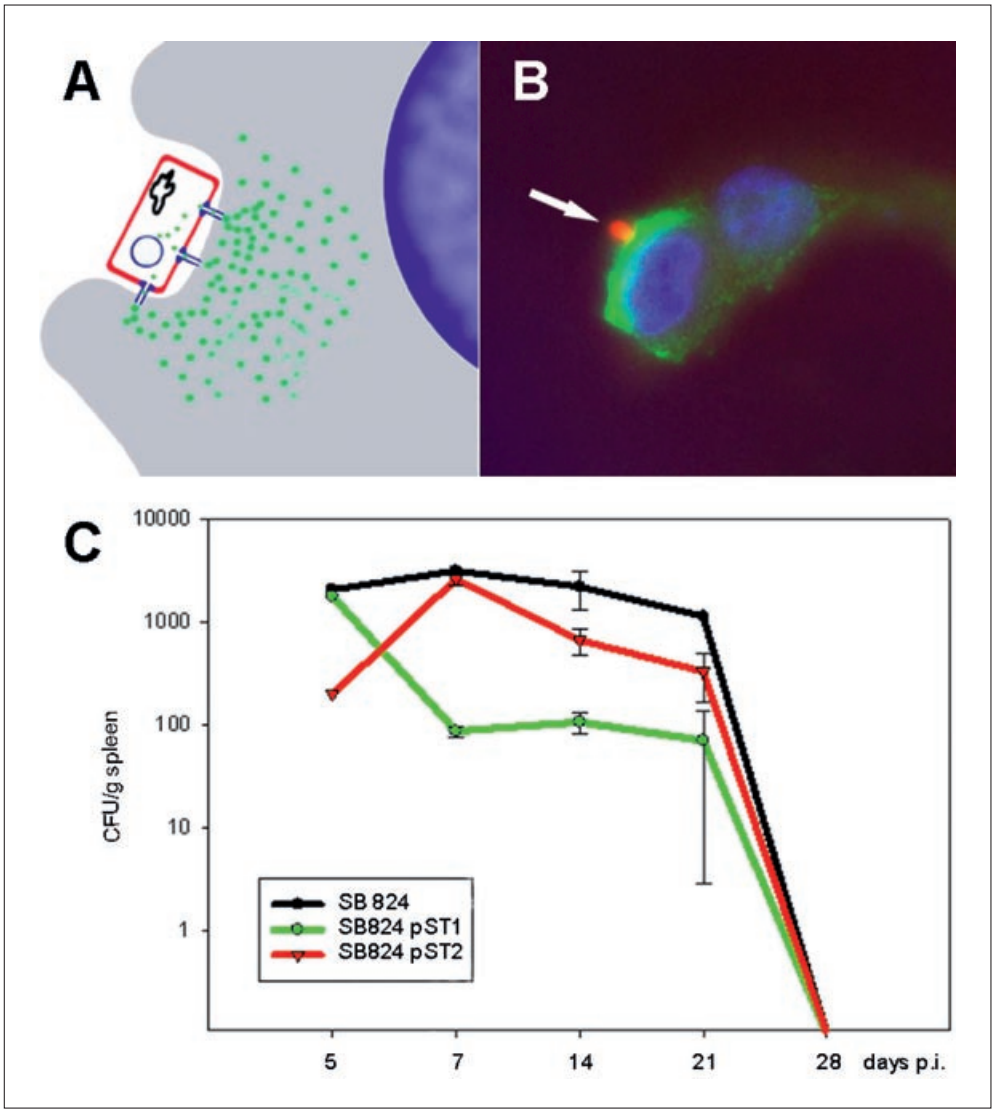

Fig. 1 Type-III secretion system dependent vaccination: (*A*) Scheme of a *Salmonella enterica* S. *Typhimurium* (red) translocating the vaccine protein (green) into the cytoplasm of a mammalian cell. (*B*) Fluorescence-microscopy of *S. enterica* pST1 (red, indicated with arrow) injecting the plasmid coded vaccine-YopE fusion protein (stained green) into the cytoplasm of a cell culture-cell (Nucleus stained blue with DAPI). (*C*) Persistence of the *Salmonella* vaccine strains (S. 824 as vector control, SB824 pST1 with plasmid coding for Vol1 and pST2 with plasmid coding for Vol2, respectively)

reduction in bacterial load, compared to control animals which received only PBS or protein buffer and adjuvant. The T3SS-dependent vaccinated mice showed some degree of protection compared with the PBS control. This protective effect is likely due to a cross reactivity of the immune responses against the vaccine carrier strain *Salmonella enterica* SB824 and *E. coli*. However, the groups mock immunized with the vaccine carrier strain SB824

and the vaccine producing strains SB824 pST1 and SB824 pST2 showed no significant difference in bacterial load 24 h after intraperitoneal challenge infection.

Taken together these results indicate that design and development of ExPEC-specific vaccines based on different virulence associated proteins is feasible. Findings of specific ExPEC antigens show that it is possible to design a vaccine for these strains despite the relative heterogeneity of the ExPEC population. These antigens constitute the basis for a preventive multi-epitope vaccine against severe infections due to extraintestinal *E. coli* strains. The exploitation of bacterial Type-III secretion system for vaccine delivery leads to prominent cellular immune response. An even more effective application route for the ExPEC vaccine is the intranasal immunization, which resulted in both high humoral and cellular immune responses. Both vaccine applications turned out to be highly protective in the peritonitis model of mice. Further investigations are in progress to improve the vaccinal efficacy of the multi-epitope ExPEC vaccine.

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Genomic *mutS-rpoS* Region Polymorphisms in Extraintestinal Pathogenic *Escherichia coli* (ExPEC) of Human and Animal Origin Correlate with Phylotypes and *In vivo* Pathogenicity

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With 2 Figures and 1 Table

Abstract

The chromosomal *mutS-rpoS* region in *E. coli* is often subjected to genetic exchange during the evolution of pathogenic lineages, and high levels of variation in this genomic region have been suggested to enforce the emergence of *E. coli* pathogens. The characterization of this genomic region in 227 extraintestinal pathogenic *E. coli* (ExPEC), and 103 commensal and environmental *E. coli* strains of human and animal source by PCR revealed a great variability of the upstream *fhlA-mutS* and the downstream *o454-nlpD* region underlining the high genetic and phylogenetic diversity within these groups of strains. One distinct pattern could be designated as "pathogenic *mutS-rpoS* pattern", as irrespective of their host origin and clinical history *E. coli* strains exhibiting this pattern possessed high numbers of virulence associated genes and besides were highly pathogenic in a chicken infection model as compared with strains harbouring other *mutS-rpoS* patterns. Thus, our data give strong evidence for a direct link between the mosaic structure of this genomic region not only with the virulence gene profile and phylogenetic background of a strain but, what is of utmost importance, with its *in vivo* pathogenicity. The *mutS-rpoS* region could therefore be of great value in identifying highly extraintestinal virulent strains among the mixed population of *E. coli* promising to be the basis of a future typing tool for ExPEC and their reservoir.

Zusammenfassung

Die chromosomale mutS-rpoS-Genregion in *E. coli* ist im Zuge der Evolution pathogener Stämme häufig einem genetischen Austausch ausgesetzt, und eine starke Variation innerhalb dieser Region wird als Schlüsselereignis in der Entstehung pathogener *E. coli* diskutiert. Die Charakterisierung dieser genomischen Region in 227 extraintestinal pathogenen *E. coli* (ExPEC) sowie 103 Kommensalen und Umweltisolaten menschlicher und tierischer Herkunft offenbarte eine große Variabilität der stromaufwärts gelegenen *fhlA-mutS* sowie der stromabwärts gelegenen *o454-nlpD*-Region, was die hohe genetische und phylogenetische Diversität innerhalb dieser Gruppe von Stämmen unterstreicht. Ein bestimmtes Muster konnte als „pathogenes *mutS-rpoS*-Muster" identifiziert werden. Unabhängig von der Wirtsherkunft sowie des klinischen Vorberichtes besaßen *E. coli*-Stämme mit diesem Muster eine höhere Anzahl Virulenz-assoziierter Gene und waren zudem im Vergleich zu Isolaten mit anderen *mutS-rpoS*-Mustern hoch pathogen im Hühner-Infektionsmodell. Somit erlauben unsere Daten einen deutlichen Hinweis auf eine direkte Verbindung der Mosaikstruktur dieser genomischen Region nicht nur mit dem Virulenzgenprofil und dem phylogenetischen Hintergrund eines Stammes, sondern auch mit seiner Pathogenität *in vivo*. Aus diesem Grund ist die *mutS-rpoS*-Region von enormer Bedeutung bei der Identifizierung hoch virulenter, extraintestinal pathogener Stämme innerhalb der gemischten Population von *E. coli* und verspricht somit die Basis für eine zukünftige Typisierungsmethode für ExPEC und deren Reservoir darzustellen.

1. Introduction

Currently, three major extraintestinal pathogenic *E. coli* (ExPEC) pathovars [uropathogenic (UPEC), newborn meningitis (NMEC), and avian pathogenic (APEC) *E. coli*] are distinguished based on clinical symptoms and virulence features. Multi locus sequence typing (MLST) data are clustering ExPEC into different phylogenetic complexes, including sequence type (ST) complex 95, 23 and 73, as well as ST62 and ST117. Despite extensive virulence typing of representative ExPEC strains, no clear correlation exists, linking the three pathovars with virulence types.

It has been recently shown that the chromosomal *mutS-rpoS* region in *E. coli* is often subjected to genetic exchange during the development of pathogenic lineages, probably contributing to the emergence of *E. coli* pathogens (Culham and Wood 2000, Herbelin et al. 2000). This active region of genomic evolution is anchored by the genes *mutS*, which encodes one of the four proteins required for the methyl-directed mismatch repair system of *E. coli*, and *rpoS*, encoding a sigma factor (σ^{38}) that regulates many stationary phase and environmental stress response genes (Ferenci 2003, Hengge-Aronis et al. 1996). The aim of this study was therefore to characterize the genetic diversity of the *mutS-rpoS* genomic region in ExPEC of human and animal source and of avian-derived faecal and environmental *E. coli* isolates to delineate associations between the polymorphism in this genomic region and the virulence gene pattern, pathogenicity and phylogeny of the strains.

2. Materials and Methods

2.1 Bacterial Strains

The genomic *mutS-rpoS* region was characterized in a total of 330 *E. coli* strains. Avian pathogenic *E. coli* (n = 138) were derived from poultry with systemic infections, uropathogenic strains (n = 32) from urinary tract infection in human (n = 21) and animals (n = 11) and new born meningitis *E. coli* strains (n = 25) from clinical cases of meningitis. Faecal (n = 70) and environmental (n = 33) samples originated from cloacal swabs from clinically healthy chickens and chicken coops.

2.2 Polymerase Chain Reactions

PCR assays for the characterization of the *mutS-rpoS* genomic region were performed according to standard protocols. For the amplification of the 5′- and 3′- sites of the *mutS-rpoS* genomic region oligonucleotide primers

- A [*fhlA*-FP] (5′ – AGAGTTCCGTAGCGATCTC – 3′),
- B [*mutS*-RP] (5′ – CTGCTGCATCATGGGCGTAT – 3′),
- C [*o454*-FP] (5′ – AAGCCCTTGCCAACATGCTAC – 3′),
- D [*nlpD*-RP] (5′ – CATAACGACACAATGCTGGTCC – 3′) and
- E [*slyA*-FP] (5′ – TTGAGTGCAGAAGAGCAGG – 3′)

were used (Fig. 1). The presence of 34 ExPEC-related virulence associated genes, related to adhesion: *afa/draB*, *crlA*, *fimC*, *hrlA*, *iha*, *mat*, *papC*, *sfa/foc*, *tsh*; iron acquisition:

Strain/Acc.-No.	*fhlA-nlpD*-region	Primer A/B	Primer C/D	Primer E/D
K-12: U29579	14.417 bp	1.208 bp	1.319 bp	no product
CFTO73: NC_004431	16.969 bp	1.673 bp	3.685 bp	no product
APEC_O1: CP000468	16.928 bp	1.673 bp	3.685 bp	no product
EPEC (RW1374)	appr. 17.000 bp	1.208 bp	4.546 bp	1.393 bp
EDL933: NC_002655	11.255 bp	1.208 bp	no product	1.393 bp

Fig. 1 Primer locations and amplicon sizes of the 5′- and 3′-borders utilized for characterization of the *mutS-rpoS* genomic region in *Escherichia coli*

chuA, *fyuA*, *ireA*, *iroN*, *irp2*, *iucD*, *sit* ep., *sit* chr.; serum resistance/protectins: *cvi/cva*, *iss*, *kpsMTII*, *neuC*, *ompA*, *traT*; toxins/hemolysins: *astA*, *cnf*, *sat*, *vat*, *hlyA*; and invasion: *ibeA*, *gimB*, *tia*, as well as genes *malX*, *pic* and *pks*, coding for a polyketide megasynthase (Nougayréde et al. 2006), was investigated using previously published multiplex PCR protocols (Ewers et al. 2007).

2.3 Phylogenetic Analyses

E. coli strains were classified according to the ECOR system established by Herzer et al. (1990) by use of a rapid phylogenetic grouping PCR technique (Clermont et al. 2000). Multi locus sequence typing was done by a scheme published by Wirth et al. (2006). Allele sequences were allocated to publicly database at the MPI for Infectious Biology, Berlin (http://web.mpiib-berlin.mpg.de/mlst). Phylogenetic clustering was performed by calculating a minimum spanning tree (MST) by means of a graphical software tool implemented in BioNumerics 4.60 (Applied Maths, Belgium).

2.4 Animal Infections

Four to eight five-week old SPF chickens per group were infected intra-tracheally with approximately 10^9 CFU of respective bacterial strains. Twenty-four hours after infection chickens were euthanized, an organ lesion score was documented and the CFU/g organ was determined as decribed previously (Antão et al. 2008).

3. Results

The characterization of the *mutS-rpoS* genomic region in ExPEC and non pathogenic avian-derived *E. coli* strains by PCR identified two structures of the upstream *fhlA-mutS* (1.200 or 1.673 bp) and four structures of the downstream *o454-nlpD* (1.319, 3.685, 4.546 bp or

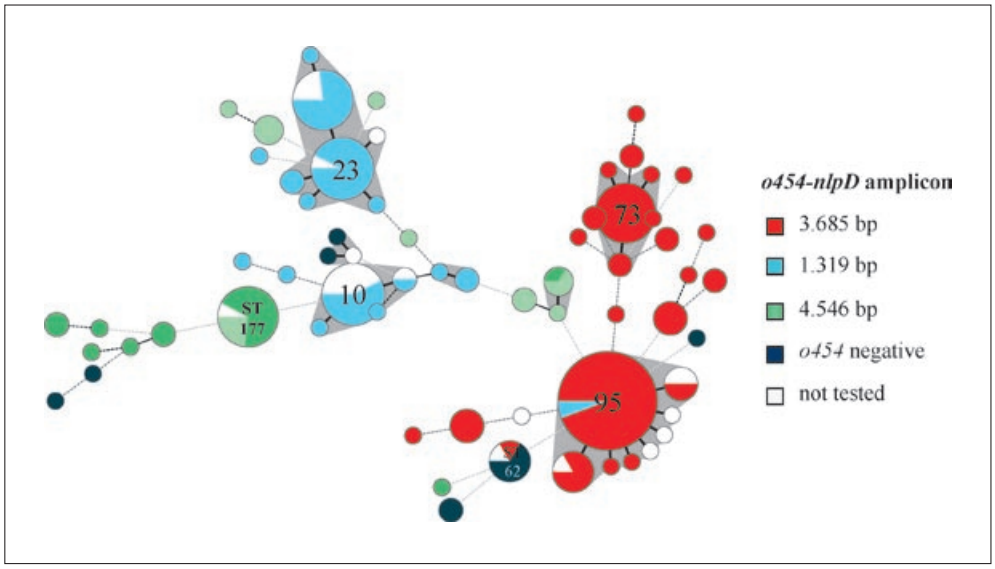

Fig. 2 Association of genomic *mutS-rpoS* region in ExPEC (n = 196) to Multi locus sequence types depicted in a Minimum spanning tree

no product) region all of which occurred in seven different combinations. Irrespective of the pathovar and host, clinical isolates predominantly harbored an *fhlA-mutS* fragment of 1.673 bp combined with an *o454-nlpD* fragment of 3.685 bp (49.4%), the so called "pathogenic *mutS-rpoS* pattern". Faecal and environmental strains mainly possessed combinations of a 1.208 bp *fhlA-mutS* and a 4.546 bp (44.6%) and 1.319 bp *o454-nlpD* amplicon (23.3%), respectively, while only 8.7% of these isolates harbored the "pathogenic pattern".

Among 34 virulence associated genes (VGs) tested, ExPEC strains with a so called "pathogenic *mutS-rpoS* pattern" harbored 19.8, whereas strains belonging to other patterns possessed 12.6 genes on average. Likewise, faecal and environmental samples with the "pathogenic *mutS-rpoS* pattern" had a mean number of 15.8 VGs compared to 7.5 to 10.8 identified in strains possessing other patterns.

Significant associations were found between the "pathogenic *mutS-rpoS* pattern" and the presence of K1 (60.2% pos.), iron related genes *chuA*, *irp2*, *iucD*, *ireA*, *sit* ep., and *sit* chr. (78.9% pos.), and the *pks*-island (43.0% pos.).

We also found a clear association of different genomic structures with certain phylotypes, with the "pathogenic pattern" being mainly grouped into ST95-Cplx (50.7%) and ST73-Cplx (15.9%) (Fig. 2), and into phylogenetic group B2 (85.4%).

Chicken infection studies showed that avian-derived strains with the "pathogenic *mutS-rpoS* pattern" revealed the strongest pathogenic potential as indicated by more severe clinical symptoms, higher bacterial loads in internal organs and organ lesion scores as compared to strains bearing other *mutS-rpoS* patterns; moreover, these strains were regularly re-isolated from the brain, indicating their invasive potential (Tab. 1).

Tab. 1 Ability of *E. coli* strains with different *mutS-rpoS* genetic regions to colonize respiratory organs and to invade internal organs of 5-week old chickens 24 h after intra-tracheal infection of 10^9 CFU bacteria and mean organ lesion scores.

Strain	Source	Serotype/ O-type	MLST (ST/Cplx)	ECOR group	*fhlA-mutS* amplicon	*o454-nlp D* amplicon	Mean No. of bacteria (CFU/g organ)				Lesion scores[1]
							Lung	Spleen	Kidney	Brain	
IMT 5155	chicken, septicemia	O2:K1:H5	140/95	B2	1.673 bp	3.685 bp	7.1×10^7	3.9×10^5	1.4×10^6	6.4×10^3	8.0 ± 2.4
IMT 9579	chicken, colisepticemia	O1	95/95	B2	1.673 bp	3.685 bp	2.8×10^6	4.0×10^5	3.1×10^5	1.3×10^3	6.1 ± 1.1
IMT 2543	chicken, septicemia	O152:H10	93/168	B2	1.673 bp	3.685 bp	5.9×10^7	2.6×10^5	4.3×10^6	6.0×10^2	5.4 ± 2.1
IMT 10740	chicken coop, air	O2:H6	new/none	B2	1.673 bp	3.685 bp	1.9×10^8	1.4×10^4	3.5×10^6	5.2×10^2	6.2 ± 1.9
IMT 5215	chicken, colisepticemia	O5:H10	n. t.	B2	1.200 bp	1.319 bp	8.0×10^6	1.8×10^4	5.8×10^3	0	3.0 ± 1.4
IMT 2283	chicken, septicemia	Osp:H6	23/23	A	1.200 bp	1.319 bp	7.6×10^8	5.5×10^4	9.7×10^3	1.8×10^1	3.1 ± 2.5
IMT 11327	healthy chicken, faecal swab	Ont:H16	295/none	B1	1.200 bp	4.546 bp	1.9×10^7	1.6×10^2	8.2×10^3	0	1.4 ± 1.3
IMT 10666	chicken coop, air	O59:H21	58/155	B1	1.200 bp	4.546 bp	5.9×10^7	1.3×10^2	3.4×10^3	0	3.5 ± 0.7
IMT 12205	healthy chicken, faecal swab	n. t.	n. t.	B1	1.673 bp	4.546 bp	5.8×10^5	3.3×10^1	4.0×10^1	0	4.2 ± 1.9

[1] Score values for severity of organ lesions ± standard deviation; the maximum additive score for lung, liver, heart, spleen and air sacs is 14;
n. t. = not tested

4. Conclusion

The grouping of ExPEC strains based on their *mutS-rpoS* pattern is basically in accordance with other genotype-based methods, like Multi locus sequence and ECOR-typing. Likewise, significant associations were observed between the *mutS-rpoS* pattern and virulence-associated features encoded by parts of the flexible genome, including the presence of K1, iron acquisition genes and polyketide synthase gene *pks*. Moreover, results of animal infection trials with APEC strains harboring different *mutS-rpoS* regions provided strong evidence for a direct link between the mosaic structure of the *mutS-rpoS* genomic region and pathogenicity suggesting this genomic region to be of great value in identifying highly extraintestinal virulent strains among the mixed population of *E. coli*.

Further characterization of the *mutS-rpoS* region in ExPEC strains as well as polymorphisms of their respective genes promise to be the basis of a future typing tool for ExPEC and their reservoir.

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Contribution of Sigma Factors RpoS and RpoE to Virulence of Avian Pathogenic *Escherichia coli*

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With 1 Figure and 1 Table

Abstract

The role of Sigma factors RpoS (σ^{38}), a stationary-phase RNA subunit that globally controls the expression of a large number of genes involved in cellular responses to stress and RpoE (σ^{24}), primarily acting as a repair system for heat denatured proteins, in the virulence of avian pathogenic *E. coli* (APEC) was studied by cell culture assays, animal experiments and *real time* RT PCR studies. The deletion of *rpoS* in the prototype APEC strain IMT5155 (O2:K1:H5), representing a strain of phylotype ST140, ST complex 95, resulted in a decreased *in vitro* adherence, while the *in vivo* pathogenicity for 5-week old chickens was not affected. In contrast, IMT5155Δ*rpoE* almost completely lost its ability to adhere to MDCK cells as well as its virulence *in vivo*. Most notably, in contrast to published results with Δ*rpoE E. coli* laboratory strains the deletion of *rpoE* in the wild type strain was not lethal. *Real time* PCR studies indicate that the two sigma factors directly or indirectly regulate a number of virulence associated genes, including those involved in bacterial adhesion and iron acquisition. Our data show that RpoS and RpoE play a fundamental role in the virulence and pathogenicity of avian pathogenic *E. coli* strains urging further investigations in the future.

Zusammenfassung

Die Bedeutung der Sigma-Faktoren RpoS (σ^{38}), einer RNA-Untereinheit, die in der stationären Phase die Expression einer Vielzahl von Stressgenen global kontrolliert, und RpoE, das in erster Linie als Reparatursystem für Hitze-denaturierte Proteine fungiert, für die Virulenz von aviär-pathogenen *E. coli* (APEC) wurde mittels Zellkultur-Assays, tierexperimentellen Versuchen und *Real-Time*- RT-PCR-Analysen untersucht. Die Deletion von *rpoS* in dem Prototyp-APEC-Stamm IMT5155 (O2:K1:H5), der einen Stamm des Phylotyps ST140, ST-Komplex 95 repräsentiert, resultierte in einer verminderten Adhärenz *in vitro*, während die *In-vivo*-Pathogenität für 5 Wochen alte Hühner unbeeinträchtigt blieb. Im Gegensatz dazu hat IMT5155Δ*rpoE* sowohl die Fähigkeit, an MDCK-Zellen zu adhärieren, als auch die Virulenz *in vivo* vollständig verloren. Von besonderem Interesse ist die Tatsache, dass entgegen publizierter Daten von Δ*rpoE E. coli*-Laborstämmen die Deletion von *rpoE* im Wildtypstamm nicht letal war. Die Untersuchungen mittels *Real-Time*-PCR weisen darauf hin, dass die beiden Sigma-Faktoren direkt oder indirekt eine Vielzahl Virulenz-assoziierter Gene, inklusive solcher, die an der bakteriellen Adhäsion und Eisenakquirierung beteiligt sind, regulieren. Unsere Daten zeigen, dass RpoS und RpoE eine entscheidende Rolle in der Virulenz und Pathogenität aviär-pathogener *E. coli* spielen, die in zukünftigen Studien ausführlicher beleuchtet werden sollte.

1. Introduction

The alternative sigma factor RpoS is recognized as a stationary-phase RNA subunit that globally controls the expression of a large number of genes involved in cellular responses

to stress (Hengge-Aronis 2002). Sigma 24 (RpoE), originally identified as a factor required for survival on exposure to extremely high temperatures, acts primarily as a repair system for denatured proteins (De Las Penas et al. 1997). Despite extensive knowledge on the role of sigma factors in *E. coli* laboratory strains, limited studies account for the contribution of sigma factors to the pathogenicity of wild type isolates. In this study the sequence of the *rpoS* and *rpoE* regulons in avian pathogenic *E. coli* (APEC), the causative agent of fatal systemic infection in poultry, were analyzed.

To ascertain the role of sigma factors RpoS and RpoE in the virulence of APEC, knockout mutants of the encoding genes of the highly virulent APEC strain IMT5155 (O2:K1:H5), representing one of the most common phylotype (ST140, ST-complex 95) of this pathovar, were generated and employed in cell culture assays, animal experiments, and *real time* PCR studies.

2. Materials and Methods

2.1 Bacterial Strains

APEC strain IMT5155 (O2:K1:H5 ST140, ST-Cplx 95, ECOR group B2), isolated from an outbreak of systemic *E. coli* infection in a chicken flock, was used for genetic manipulations and following functional assay (Ewers et al. 2004b). *E. coli* strain IMT11327 (Ont:H16; ST295; ECOR group B1), isolated from the faeces of a clinically healthy chicken, was included as control strain in cell culture assays and chicken infection experiments. While IMT5155 harbors many of the so far known ExPEC-related virulence genes (*crlA*, *fimC*, *tsh*, *mat*, *chuA*, *fyuA*, *ireA*, *iroN*, *irp2*, *iucD*, *sit*, *cvi/cva*, *iss*, *neuC*, *kpsMTII*, *ompA*, *traT*, *vat*, *ibeA*, *gimB*, and *malX*) the faecal strain IMT11327 was tested positive for *crlA*, *fimC* and *ompA* only (Ewers et al. 2007).

2.2 Cell Culture Assays

Adhesion tests were performed according to a previously published protocol (Ewers et al. 2004a) using MDCK-1 (Mardin-Darby canine kidney) cells infected with a multiplicity of infection (MOI) of 100 bacteria per epithelial cell. Cell culture assays were performed in duplicate and were independently repeated at least three times. Results are expressed as the averages of all replicate experiments ± standard deviations. Graphical presentations and statistical calculations were performed using the Statistical Package for the Social Sciences (SPSS, version 15.0).

2.3 Bacterial Manipulation

Knockouts of the *rpoS* and *rpoE* genes in *E. coli* strain IMT 5155 were generated by using the method of Datsenko and Wanner (2000).

2.4 Animal Infection Tests

Five-week old SPF chickens were infected intra-tracheal with 10^9 bacteria of the respective wild type and mutant strains. Twenty-four hours after infection chickens were eutha-

nized; organs were aseptically removed and homogenized to determine the CFU/g organ after an organ lesion score has been determined as described previously (ANTÃO et al. 2008) (Tab. 1).

2.5 Real-Time RT PCR

Real-time PCR assays were performed with *in vitro* cultured bacterial strains using the SYBR-Green method (LightCycler® FastStart DNA Master SYBR Green I, Roche Applied Science, Basel, Switzerland). Results were analyzed using the LightCycler 2.0 software and were normalized against housekeeping gene dnaB. Each experiment included three technical repeats. Primers for genes included in Real-time PCR assays (*fimC*, *tsh*, *iss*, *cvi/cva*, *sitA*, *iutA*, *chuA*, *ireA*, *fyuA*, *iroN*, *fur*, *tonB*, *vat*, *ibeA*, *ibeB*, *ompA*, and *gimB*) were designed manually and quality checked by Oligo Analyzer version 3.0 (IDT SciTools).

3. Results

Knockout mutants of *rpoS* and *rpoE* in APEC strain IMT5155 were successfully generated and showed no significant growth impairment in Luria Bertani broth as compared to the wild type strain. The ability of IMT5155Δ*rpoE* to adhere to MDCK cells was reduced to 2.4 % compared to the wild type strain 3 h after infection while IMT5155Δ*rpoS* adhered 3.5-fold less than the wild type strain (Fig. 1).

In vivo infection experiments showed that the virulence of IMT5155Δ*rpoE* was almost completely lost in 5-week old chickens after intra-tracheal infection of 10^9 bacteria. Compared to the wild type strain there was a decrease in bacterial numbers in internal organs up to a 1000fold when infected with the *rpoE* mutant, while no bacteria were isolated from the brain which was quite comparable to the data of the faecal non pathogenic strain IMT11327. In contrast, the *rpoS*-deletion in IMT5155 did not result in significant differences of bacterial loads in internal organs and mean organ lesion scores as compared to the wild type strain and caused strong clinical symptoms in chickens, typical of a systemic *E. coli* infection. Bacteria were re-isolated in numbers similar to the wild type strain from all internal organs, including the brain (Tab. 1).

Tab. 1 Ability of *E. coli* wild type, mutant and faecal strain to colonize respiratory organs and to invade internal organs of 5-week old chickens and mean organ lesion scores 24 h after intra-tracheal infection of 10^9 CFU bacteria.

Strain	Mean no. of bacteria (CFU/g organ)[1]				Mean organ lesion scores[2]					
	Lung	Spleen	Kidney	Brain	Air sacs	Lung	Heart	Liver	Spleen	Total
IMT 5155	9.2×10^7	3.5×10^5	6.4×10^6	5.7×10^2	1.7 ± 0.9	2.5 ± 0.9	1.2 ± 1.1	0.3 ± 0.5	0.9 ± 0.2	6.6 ± 3.6
5155Δ*rpoS*	7.6×10^7	1.5×10^6	1.9×10^6	1.9×10^2	1.6 ± 0.9	2.0 ± 1.0	0.8 ± 1.0	0.2 ± 0.4	1.0 ± 0.2	5.6 ± 3.5
5155Δ*rpoE*	1.9×10^5	4.1×10^1	2.7×10^3	0	0.3 ± 0.4	1.1 ± 1.0	0.0 ± 0.0	0.1 ± 0.3	0.5 ± 0.3	2.0 ± 2.0
IMT11327	1.9×10^7	1.6×10^2	8.2×10^3	0	0.4 ± 0.5	0.6 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.5	1.4 ± 1.3

[1] The CFU/g organ was determined in 16 chickens for wild type and mutant strains and in 5 animals for faecal strain IMT11327.
[2] The mean organ lesion score was determined in 30 chickens for wild type and mutant strains and in 5 animals for faecal strain IMT11327.

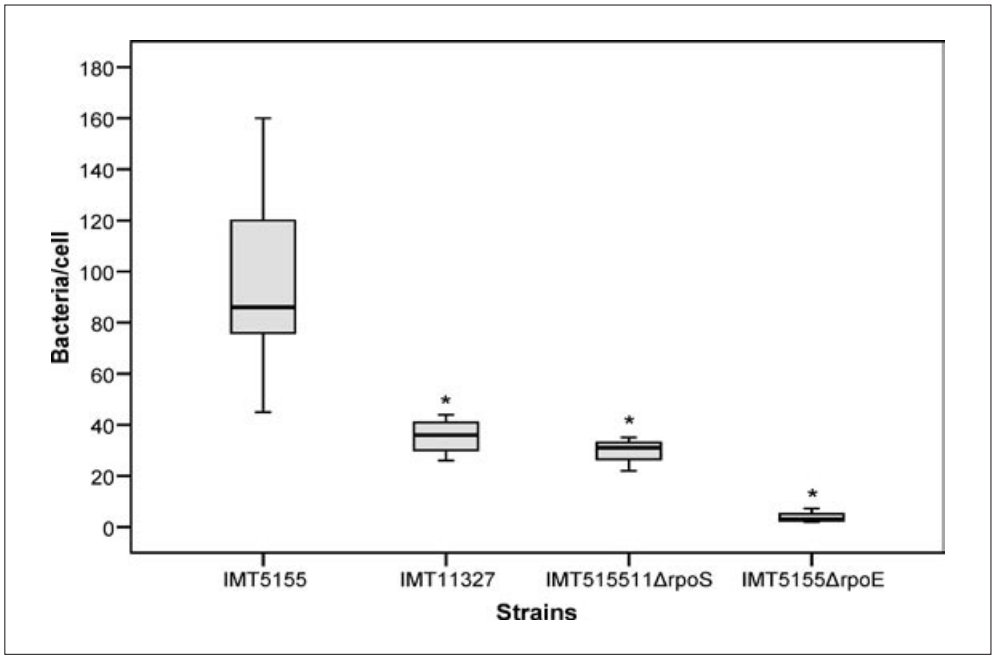

Fig. 1 Adherence of *E. coli* wild type (IMT5155), mutants (IMT5155Δ*rpoS*; IMT5155Δ*rpoE*) and non pathogenic faecal strain (IMT11327) to MDCK-1 epithelial cells at 3 h post infection with an MOI of 100.
* $p \leq 0.001$

In Real-time PCR assays IMT5155Δ*rpoS* yielded an up-regulation of *fimC* (4.9), *iutA* (4.1), *ibeA* (2.8), *iss* (1.9), and *tsh* (1.6). A significant down-regulation was observed for *fur* (8.9-fold), while other genes tested were only moderately regulated. For the *rpoE* mutant an up-regulation of *ompA* (14.1), iron-related genes *tonB* (2.8), *fur* (2.7), *chuA* (1.6), and *sitA* (1.6), toxin gene *vat* (3.0), adhesion gene *tsh* (2.3), and invasion-associated genes *ibeA* (1.4) and *ibeB* (1.8) was observed, while type 4 fimbrial gene *fimC* (2.1-fold) and ferric *Yersinia* uptake gene *fyuA* (1.8-fold) were down-regulated.

4. Conclusion

The present data show that sigma factors RpoE and RpoS play a pivotal role in the virulence and pathogenicity of avian pathogenic *E. coli* strains. Most remarkably, in contrast to published results with Δ*rpoE E. coli* laboratory strains IMT5155Δ*rpoE* was not lethal. This finding again highlighted the enormous diversity of *E. coli.* The two sigma factors were found to particularly regulate iron- and adhesion-related genes. However, there is no obvious causality between biological effects observed for sigma mutants *in vivo* and *in vitro* and regulation of virulence-associated genes. The relevance of the strong up-regulation of outer membrane gene *ompA*, involved in invasion of the brain endothelium by new born meningitis *E. coli* (Wang et al. 2002) in the *rpoE* mutant and the down-regulation of iron uptake regulator *fur* in the *rpoS* mutant are only two effects that remain to be investigated.

The deletion of *rpoE* in APEC strain IMT5155 leads to an almost complete loss of virulence and adhesion ability in a chicken infection model and canine epithelial cells, respectively. In the case of the *rpoS* mutant, a decreased *in vitro* adherence was observed, while this mutant did not differ significantly in its virulence *in vivo*. However, these effects are not due to growth changes *in vitro*. Complementation assays and screening for well known compensatory mutations especially in the *rpoE* mutant are currently performed in our lab to validate *in vivo* and *in vitro* data.

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Host-Pathogen Interaction

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Shigella Infectious Maneuver for Circumventing Intestinal Barrier Functions

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Abstract

Shigella possesses a large (~220 kb) plasmid, on which the major virulence-associated proteins called effectors and the secretion system called type-III secretion system (T3SS) are encoded. *Shigella* secretes many effectors through the T3SS into the surrounding space and directly into the host cell cytoplasm, where the effectors play pivotal roles in promoting bacterial infection and in subverting host cell signal transduction pathways and the innate immune response. Here I highlight recent advances in our understanding of the *Shigella* infectious maneuver as an example for the pathogen possessing highly sophisticated strategies for infection of human intestinal epithelium.

Zusammenfassung

Shigella besitzt ein großes Plasmid (ca. 220 kb), auf dem die wichtigsten Virulenz-assoziierten Proteine, die sogenannten Effektoren sowie das sogenannte Sektretionssystem-Typ-III (T3SS), kodiert sind. *Shigella* sekretiert eine Vielzahl von Effektoren durch das T3SS in die Umgebung sowie direkt in das Zytoplasma der Wirtszelle, wo die Effektoren eine wichtige Rolle bei der Förderung der bakteriellen Infektion und der Zerstörung der Signalübertragungswege der Wirtszelle und der angeborenen Immunreaktion spielen. Es werden neue Entwicklungen in der Erforschung des Infektionsverhaltens von *Shigella* vorgestellt als Beispiel für ein Pathogen, das über hoch spezialisierte Infektionsstrategien für die Infektion des Darmepithels beim Menschen verfügt.

Shigella are a member of pathogenic *Escherichia coli*, and a highly adapted human pathogen, that cause bacillary dysentery (shigellosis). Shigellosis has been ranked as a major leading infectious killer in developing countries where ~0.5 million people, those are mostly under 5 years, die of the disease every year. As *Shigella* efficiently transmits person-to-person via the fecal-oral route by ingesting a very small number of bacteria such as 10–100 bacteria, the control of shigellosis under low sanitary condition is not easy. *Shigella* infection of the intestinal epithelium begins with bacterial entry into the M cells that overlie solitary lymphoid nodules of colon. Once they reach underneath the M cells, bacteria invade the resident macrophages, escape from the phagosomes, multiply within the cytoplasm, and induce rapid cell death. Meanwhile, the bacteria enter the surrounding enterocytes through the basolateral surface by inducing large membrane ruffles. After the bacterial entry into the cytoplasm, *Shigella* multiply and move within the cytoplasm as well as into adjacent epithelial cells by inducing actin polymerization at one pole of bacterium. During multiplication

in epithelial cells, *Shigella* release peptidoglycan (PGN) and lipopolysaccharide (LPS) into the cytoplasm. These bacterial components are recognized by Nod-like receptors and Toll-like receptors, which stimulate inflammatory signaling cascades and induce cellular and humoral immune responses. The intracellular activities of *Shigella* and the host inflammatory response thus finally result in destruction of intestinal barrier integrity, which eventually leads to shigellosis (Sansonetti and Di Santo 2007, Ogawa et al. 2008).

The intestinal epithelium possesses at least four major barrier functions; the integrity of the epithelial barrier; the rapid cell turn over; the innate immune response; and the resident commensal bacterial flora. Nevertheless, *Shigella* (and many other pathogenic bacteria) are capable of circumventing the intestinal defense barriers and exploit the intestinal epithelium as the replicative foothold. During infection of intestinal epithelium, *Shigella* deliver many numbers of effectors (perhaps more than 50) via the T3SS to the surrounding space and host cell cytoplasm, in which the bacterial effectors involve in various infectious aspects such as the invasion of macrophages and epithelial cells, cell-to-cell spreading, suppression of host inflammatory response, evasion of autophagic recognition, and down regulation of rapid epithelial turn over.

1. Bacterial Entry into Host Intestinal Epithelium

Shigella (and *Salmonella*) are capable of inducing macropinocytic events from epithelial cells via production of large membrane ruffles around the bacteria entry site, by which the pathogens efficiently enter the epithelial cells. *Shigella* exploit the interactions between a subset of T3SS-delivered effectors and their host target proteins (or target functions) to stimulate several host cell signaling pathways that are involved in inducing actin polymerization and remodeling the architecture of the epithelial cell surface. For example, IpaB, which is secreted around the bacterial surface, can interact with CD44 receptor and stimulate the basolateral entry of polarized epithelial cells. IpaC, which is delivered around the bacterial surface, interacts with β1-integrin, and the cytoplasmic delivered IpaC integrates into the plasma membrane can stimulate actin polymerization (Nhieu et al. 2005). IpgB1 specifically interacts with ELMO and stimulates Rac1 via the ELMO-Dock180 pathway, thus leading to large membrane ruffles (Handa et al. 2007). IpgD has phosphatidylinositol 5-monophosphate, thereby promoting local actin nucleation. IpaA binds vinculin head and β1-integrin cytoplasmic domains, which stimulates RhoA, thus inducing the loss of actin stress fibers (Nhieu et al. 2005). VirA, a member of VirA/EspG family distributing among enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *Shigellla*, share the activity to induce degradation of local microtubules (MTs) (Yoshida et al. 2002). VirA is delivered into the host cell cytoplasm near the site of bacterial entry thus induces local membrane ruffles via the cross-talk between RhoA and Rac1 (Ogawa et al. 2008).

2. Cell-To-Cell Movement

Burkholderia pseudomallei, *Listeria monocytogenes*, *Mycobacterium marinum*, *Rickettsia conorii* and *Shigella* are capable of inducting actin nucleation at one pole of bacterium to gain the propulsive force required for the movement within the host cells as well as into

the neighboring cells, called actin-based bacterial motility. *Shigella* conduct the activity via VirG (IcsA), an outer-membrane protein, since VirG specifically interacts with one of the WASP family, N-WASP. Upon interaction of VirG with N-WASP, N-WASP becomes activated, which, in turn, recruits and activates the Arp2/3 complex, thus leading to actin polymerization. Some of the motile bacterium impinge on the host plasma membrane and protrude filopodium, and finally penetrate into that of neighboring cells, thus allowing the motile bacterium to move into adjacent cells (Ogawa et al. 2008). For escaping from the double membranes surrounding the bacterium, IpaB delivered from motile *Shigella* via the T3SS plays a major role (Nhieu et al. 2005).

It has recently been indicated that *Shigella* movement within the host cytoplasm is physically hindered by MTs, but a motile *Shigella* can circumvent the problem by destroying surrounding MTs by secreting VirA. Biochemical analysis showed that VirA possesses some affinity to a tubulin heterodimer and activity to induce MTs degradation (Yoshida et al. 2002, 2006). Indeed, intracellular *virA* mutant shows less capability to move smoothly within the epithelial cytoplasm than the wild type, thus becoming less capable of cell-to-cell spreading and attenuated in mice infection (Yoshida et al. 2006). These studies have indicated that the VirA effector has dual roles in both bacterial invasion and cell-to-cell movement.

3. Suppression of Host Inflammatory Signaling

Recent studies have indicated that mucosal pathogenic bacteria, such as *Shigella*, EPEC, EHEC, *Samonella* and *Yersinia*, possess some activity to down regulate host inflammatory responses during infection. As mentioned above, *Shigella* infection of macrophages and epithelial cells causes sever inflammation, since during multiplication *Shigella* release the LPS and PGN into host cytoplasm, and the bacterial components stimulate signaling cascades that involve mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB, thus leading to production of proinflammatory cytokines and chemokines (Sansonetti and Di Santo 2007). In order to suppress the inflammatory responses, *Shigella* deliver many numbers (perhaps more than 10) of effectors via T3SS, those include OspG, OspF and IpaH9.8 into host cytoplasm. For example, OspG, which binds to E2s, an ubiquitin-conjugated enzyme, such as UbcH5b, a component of the Skf1-Culin-F-box protein $(SCF)^{\beta\text{-}TrCP}$ complex that is involved in phospho-IκBα ubiquitination and its subsequent degradation by the proteasome. Thereby, OspG can inhibit IκBα degradation required for the NF-κB activation (Kim et al. 2005). OspF, which is translocated into the host cell nucleus, possesses a specific phosphatase activity which causes dephosphorylation and inactivation of MAPKs, such as ERK1/2, JNK and p38, thereby interfering with the phosphorylation of the critical serine residue of histone H3 activity, required for the transcription of NF-κB-regulating subset of genes (Arbibe et al. 2007). IpaH9.8 is delivered into the host cell cytoplasm and nucleus. The IpaH9.8 moiety translocated to the nucleus can interact with $U2AF^{35}$, an mRNA splicing factor, and the interaction interferes with the splicing activity. Infection of mice via the nasal route by an *iapH9.8* mutant induces severer lung inflammation and greater proinflammatory cytokine production than in the wild type. Most important, the bacterial colonization rate of the *ipaH9.8* mutant in the lung tissue is greatly reduced compared with that of the wild type *Shigella* (Okuda et al. 2005), implying that the bacterial activity to down regulate the host inflammatory responses is pivotal for promoting bacterial colonization.

4. Evasion of Autophagic Recognition

During multiplication of *Shigella* within host cell cytoplasm, the bacteria are monitored by another innate defense system that is mediated by autophagy. Autophagy is a ubiquitous degradation system in eukaryotic cells that is crucial as the cellular response to starvation and stress, for removal of damaged or surplus organelles. Autophagy is a highly conserved membrane-trafficking pathway; during autophagy, an isolation membrane wraps around undesirable cytoplasmic contents by double or lamella membranes. The material, once delivered to an autophagosome, is finally degraded after fusion with lysosome. This autophagic degradation system is pivotal as an innate defense system for eliminating cytoplasmic invading microbes. In the case of *Shigella* and *L. monocytogenes*, which can multiply and move by actin-based bacterial motility within the host cell cytoplasm via activity of their respective surface-expressed proteins VirG and ActA, both are capable of escaping from autophagic recognition. In *Shigella*, VirG is recognized by a component of the autophagic pathway, Atg5, however, the IcsB effector secreted via the T3SS can camouflage the target VirG protein from Atg5 by competitively binding to the VirG portion with Atg5, thus allowing the bacteria to evade autophagy and multiply within the epithelial cells (Ogawa et al. 2005).

5. Slowdown of Rapid Epithelial Cell Turnover

The intestinal epithelium self-renews every several days, providing an important innate defense system that limits microbes' colonization. The raid turnover is sustained by the vigorous proliferation of epithelial progenitors that continuously migrate upwards from the bottom of the crypts. This epithelial renews seem to be highly dynamic in response to microbe infection. For example, in the case of *Trichuris trichuria*, a cecal-dwelling parasitic nematode, the rate of epithelial turnover in murine large intestines can be increased in response to the nematode infection (Cliffe et al. 2005). Under the situation, many pathogenic bacteria, including *Shigella*, are capable of efficiently colonizing the epithelium. It has recently reported that *Shigella* have some tactics to slowdown the rapid turnover of the epithelial progenitors (Iwai et al. 2007). Indeed, using rabbit ileal loops at various stages of infection it has been indicated that *Shigella* can directly access the intestinal crypt after inducing inflammatory responses in the intestinal tissue, since the crypts can be opened up upon inflammation. *Shigella* IpaB effector, which is known to act as the invasin and translocater of the T3SS, is delivered into the epithelial progenitor cytoplasm by intracellular bacteria and causes cell-cycle arrest by interacting with Mad2L2, an anaphase-promoting complex/cyclosome (APC/C) inhibitor. Cyclin B1 ubiquitination assay indicates that APC undergoes unscheduled activation as the consequence of the interaction of IpaB with Mad2L2. When synchronized HeLa cells are infected with *Shigella*, the cells cause cell-cycle arrest at the G2/M phase in an IpaB/Mad2L2-depedent manner. The IpaB activity is also demonstrated in rabbit intestinal crypt progenitors, and the IpaB-mediated cell arrest contributes to efficient colonization of the intestine. The bacterial activity to retard intestinal epithelial renewal thus seems to be important for gaining and prolonging the infectious foothold during infection (Iwai et al. 2007).

6. Conclusion

Studies of the *Shigella* infectious maneuver including the characterization of the T3SS-secreted effectors have been provided further insights into our understanding of the highly evolved bacterial infectious systems, that also provide lots of clue to develop new *Shigella* vaccine (Suzuki et al. 2006), animal model for shigellosis (Shim et al. 2007), and new drugs to control the illness.

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Strahlenanwendung und Strahlenforschung in Deutschland

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Seit der Entdeckung von Radioaktivität und ionisierender Strahlung am Ende des 19. Jahrhunderts hat die Nutzung dieser physikalischen Phänomene Eingang in zahlreiche Anwendungsbereiche der Medizin, der naturwissenschaftlichen Forschung und der Technik gefunden. Dennoch stößt Strahlenanwendung in der Gesellschaft auf emotionale Ablehnung und eine irrationale Strahlenangst. Der Akzeptanzmangel bewirkte in den vergangenen Jahren einen kontinuierlichen Abbau von Forschungseinrichtungen, die sich mit der Anwendung von ionisierender Strahlung in Medizin, Umwelt und Wissenschaft beschäftigen. Der Band diskutiert Entwicklungstendenzen der Strahlenanwendung bis zum Jahre 2020 und versucht, die sich daraus ergebenden Anforderungen an die Strahlenforschung zu definieren. Dabei werden neben Fragen der Strahlenbiologie, der Strahlenphysik, des Strahlenschutzes sowie medizinischer und technischer Strahlenanwendungen auch die Probleme der unfallbedingten oder beabsichtigten missbräuchlichen Radionuklidfreisetzungen mit Strahlengefährdung der Bevölkerung sowie die Notwendigkeit der Erarbeitung effektiver präventiver Strategien behandelt. Ungeachtet des zu erwartenden Wandels der Methodenvielfalt bis zum Jahre 2020 zeigen die Beiträge, dass Strahlenanwendung auch weiterhin im bisherigen Umfang stattfinden wird und eine kontinuierliche Begleitung durch adäquate Strahlenforschung erforderlich bleibt.

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Molecular and Epidemiologic Basis of Urinary Tract Infection in Women

Thomas J. Hannan and Scott J. Hultgren (St. Louis, USA)

With 4 Figures

Abstract

The rise in antibiotic resistant pathogens, emergence of new diseases, and involvement of bacterial pathogens in diseases formerly thought to be due to non-infectious agents have rekindled the need to understand the "molecular logic" of virulent bacteria. Studying uropathogenic *Escherichia coli* (UPEC) as a model system for mucosal infection, we have discovered that UPEC infection in the urinary bladder, previously considered to be solely extracellular in nature, has two critical intracellular phases. Early in infection, UPEC have been found to replicate within bladder epithelial cells to form intracellular bacterial communities in both the mouse and human. Upon resolution of acute infection, small collections of UPEC remain within bladder epithelial cells in LAMP1+ vesicles, forming quiescent intracellular reservoirs that are capable of re-emerging to cause recurrent infections. These findings are forming a foundation for the development of new strategies for the prevention and treatment of mucosal infectious disease.

Zusammenfassung

Der Anstieg von Antibiotikaresistenzen bei Krankheitserregern, das Auftreten neuer Erkrankungen und die Beteiligung bakterieller Krankheitserreger an Erkrankungen, die man früher nicht-infektiösen Erregern zugeschrieben hat, verdeutlichen, dass wir die „Molekulare Logik" virulenter Bakterien verstehen müssen. Indem wir uropathogene *Escherichia coli* (UPEC) als Modellsystem für Schleimhautinfektionen verwenden, konnten wir zeigen, dass die Infektion mit UPEC-Bakterien, von der man früher annahm, dass sie ausschließlich extrazellulär abläuft, zwei bedeutende intrazelluläre Phasen enthält. Im Frühstadium der Infektion können UPECs in Blasenepithelzellen replizieren und sowohl in Mäusen als auch im Menschen intrazelluläre Bakteriengemeinschaften bilden. Nach dem Abklingen der akuten Infektion verbleiben kleine Ansammlungen von UPECs in den Blasenepithelzellen in sogenannten LAMP+1-Vesikeln. Diese bilden ein intrazelluläres Reservoir, das zum Wiederaufleben der Infektion und dadurch zu rezidivierenden Infektionen führen kann. Diese Ergebnisse bilden die Grundlage für die Entwicklung neuer Strategien zur Verhinderung und Behandlung von Schleimhautinfektionen.

1. Introduction

The study of bacterial pathogenesis is rapidly evolving, as advances in the understanding of bacterial mechanisms of virulence and host responses to infection continue to change our perspective on infectious disease. Microbes have even been implicated in chronic inflammatory diseases and cancers previously thought to have non-infectious etiologies (Cassell 1998, Marshall and Warren 1984). However, translation of the many significant experi-

mental advances into effective clinical interventions has remained limited due to our lingering ignorance of these complex host-pathogen interactions. Furthermore, these limited gains have been met full force both by the emergence of new infectious diseases and the continued rise in antibiotic resistance among bacterial pathogens. These struggles highlight the overwhelming need to develop new animal models to study mechanisms of infectious disease as well as to more fully understand existing models in light of recent advances (VIRGIN 2007). In this presentation, we describe how our study of bacterial pili led to a paradigm shift in our understanding of the pathogenesis of urinary tract infections by uropathogenic *E. coli* in both the mouse and the human.

2. The Chaperone/Usher Pathway and Type 1 Pili

Using UPEC as a model system, we uncovered the fine details of a molecular machine, called the chaperone/usher pathway, used by diverse pathogenic bacteria to assemble adhesive fibers called pili on their surfaces (Fig. 1*A*) (SAUER et al. 2004). Pili initiate host-pathogen interactions critical in the pathogenic processes of a wide range of bacteria. These fibers assemble from pilus subunits at an outer membrane pore called the usher, which also serves as the anchor. Assembly occurs by non-covalent interactions between subunits whereby each subunit has the amino acid sequence necessary to form an incomplete immunoglobulin-like domain. We discovered that periplasmic chaperones serve as folding templates for pilus subunits, enabling them to properly fold into immunoglobulin-like domains in a mechanism called donor strand complementation that represents a surprising twist of the classic Anfinsen postulate. At the usher the N-terminal extension of the next subunit provides the β-strand necessary to complete the immunoglobulin fold in a canonical fashion, simultaneously displacing the chaperone in a process called donor strand exchange (Fig. 1*B*) (BARNHART et al. 2000, REMAUT et al. 2006, SAUER et al. 1999, 2002). Once fully assembled, we have demonstrated by atomic force microscopy that type 1 pili are capable of extending under an applied force (Fig. 1*C, D*). This extensibility is the result of the unwinding of the pilus rod's helical quaternary structure and is fully reversible (MILLER et al. 2006). Therefore, this elastic property of type 1 pili may serve as an essential mechanism for absorbing physiological shear forces encountered during urinary tract infections (UTI).

3. Type 1 Pili and Urinary Tract Infections

Type 1 pili are critical UPEC virulence factors in a murine model of cystitis (Fig. 2) (CONNELL et al. 1996, WRIGHT et al. 2007). During UPEC infection the type 1 pilus-associated adhesin, FimH, mediates adherence to and invasion of the superficial facet cells of the urinary bladder epithelium (urothelium) (MULVEY et al. 1998). Type 1 pili-mediated binding initiates signal transduction cascades in urothelial cells, enabling UPEC internalization by a zippering mechanism (MARTINEZ and HULTGREN 2002, MARTINEZ et al. 2000). UPEC binding and invasion also elicit rapid and robust innate responses by the host urothelium, such that UPEC infection may have significant implications for normal epithe-

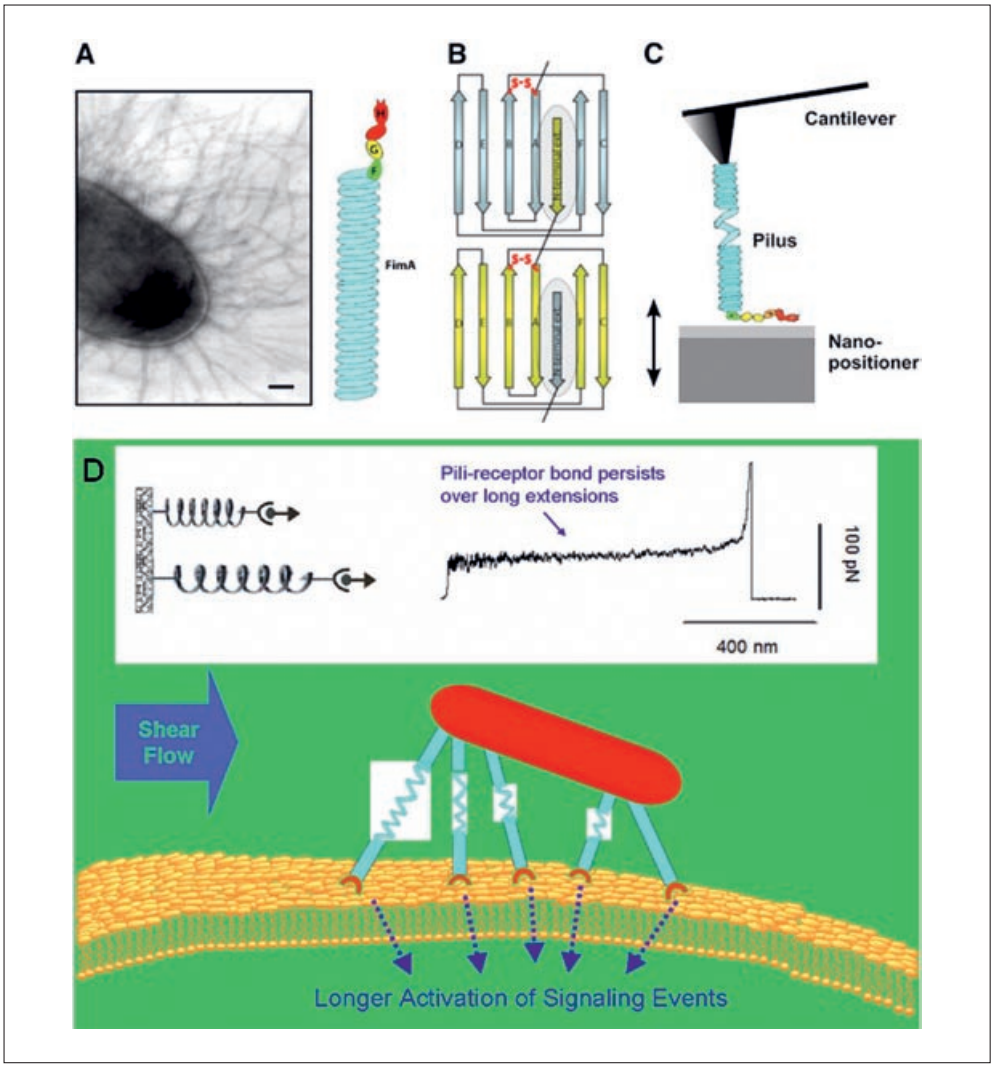

Fig. 1 (*A, left*) Negative-stain electron micrograph of uropathogenic *E. coli* (UTI89) cell expressing type 1 pili (*scale bar* = 200 nm). (*A, right*) A cartoon diagram depicting the structural subunits of type 1 pili. FimH is the adhesin at the distal end of the tip fibrillum, followed by FimG and FimF. Oligos of FimA form the helical pilus rod structure that is visible in the micrograph. (*B*) Two-dimensional diagram of donor-strand exchange between FimA Ig-like domains. Each pilin structural subunit donates its *N*-terminal extension to complete its neighbor's fold and form a protein chain. The *N*-terminal extension is held in place through non-covalent interactions (*shaded region*) such as hydrogen bonds and hydrophobic interactions. Each pilin subunit has one disulfide bond (*red*) between the A and B ß-strands, close to where the *N*-terminal extension ends. (*C*) Diagram of the single-molecule atomic force microscopy (AFM). Purified pili were adsorbed onto a glass substrate and then stretched using the tip of a cantilever. (*D*) Model for how the ability of the type 1 pilus helical rod to unravel allows prolonged binding to the host receptor in the face of shear flow. (Inset) AFM experiment demonstrating that a single type 1 pilus rod bound to its receptor can lengthen several hundred nanometers under constant tension before breaking or disassociating. (Figure and legend modified from Miller et al. 2006).

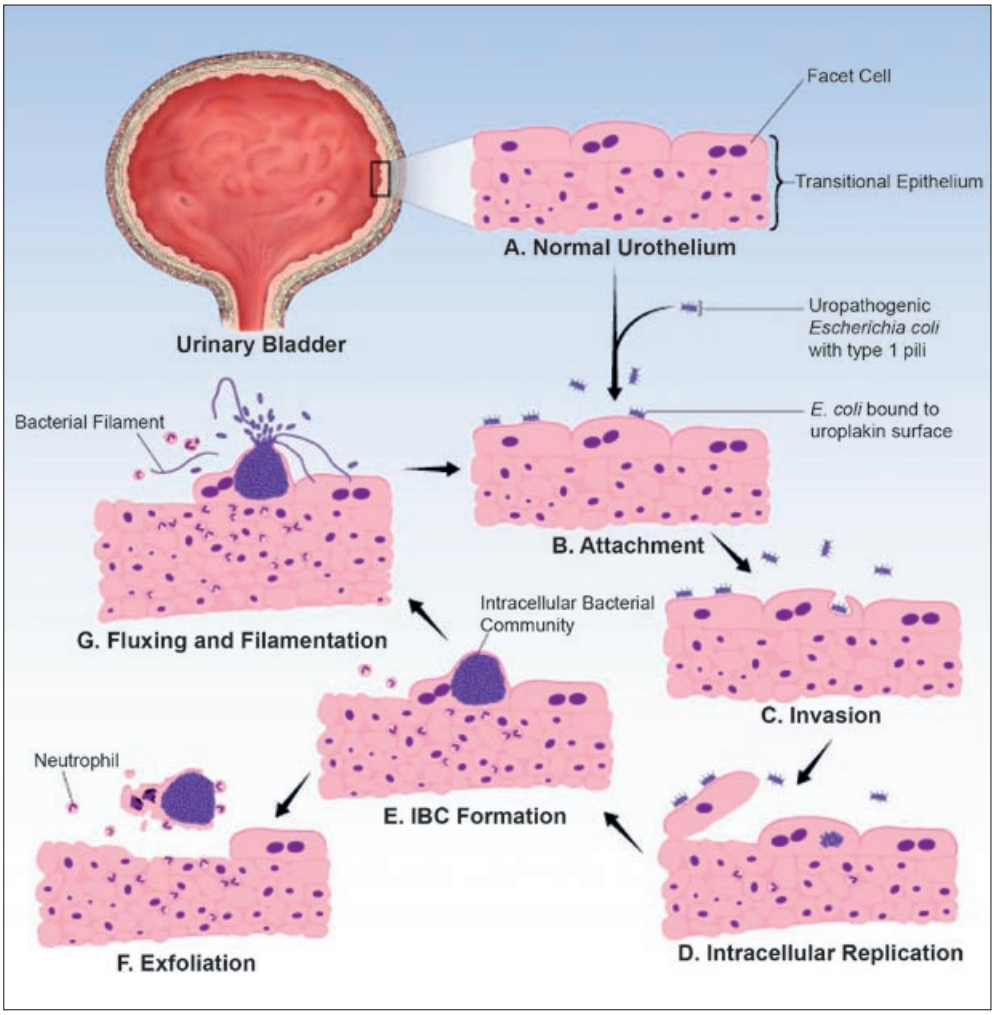

Fig. 2 Illustration of the intracellular bacterial community (IBC) cycle. (*A–B*) Uropathogenic *E. coli* adhere to normal urothelial tissue during acute infection by type 1 pilus binding to mannosylated glycoproteins such as uroplakin Ia. (*C*) Concerted type 1 pili binding enables internalization of UPEC into superficial facet cells. (*D–E*) UPEC rapidly replicate within the facet cell cytoplasm and form complex intracellular bacterial communities. (*F–G*) The host response to infection is to recruit neutrophils to the site of infection and for the facet cells to exfoliate by undergoing apoptosis. If bacterial fluxing and dispersion of the IBC does not occur prior to cell exfoliation, then the IBC may be expelled with the urine. Upon emergence of UPEC into the bladder lumen prior to urothelial exfoliation, an event often accompanied by filamentation, UPEC are able to reinvade other facet cells or colonize the underlying transitional epithelial cells (figure modified from ROSEN et al. 2007).

lial renewal and bladder cancer (MYSOREKAR et al. 2002). Recently, in collaboration with Fredrik ALMQVIST, we have discovered several small compounds that profoundly and specifically inhibit type 1 pilus biogenesis and thus have great promise as therapeutic agents against UPEC UTI (PINKNER et al. 2006).

4. The Intracellular Bacterial Community

We have found that UPEC entry into superficial facet cells of the urothelium is a critical event in acute lower urinary tract disease as the invasive bacteria form biofilms within the urothelial cells to subvert innate host defenses (Anderson et al. 2003). Entry into urothelial cells activates a complex bacterial genetic cascade leading to the formation of intracellular bacterial communities (IBCs) (Justice et al. 2004). These IBCs undergo a defined maturation and differentiation program involving the expression of type 1 pili (Wright et al. 2007). Within hours, bacteria begin to flux away from the IBC and emerge from the dying urothelial cells, often in filamentous form, into the lumen to colonize and invade neighboring cells (Justice et al. 2004). Deletion of the cell division inhibitor gene, *sulA*, has demonstrated that UPEC filamentation is necessary for virulence after the first round of IBC formation in the immunocompetent host (Justice et al. 2006).

5. Evidence of Intracellular Bacterial Communities in Human Urinary Tract Infection

We have translated our studies of acute UPEC pathogenesis in the mouse to address the problem of recurrent cystitis in women. We found evidence of IBCs in human patients with lower UTI (Fig. 3) (Rosen et al. 2007). IBCs were found in 14 of 80 (18 %) urines

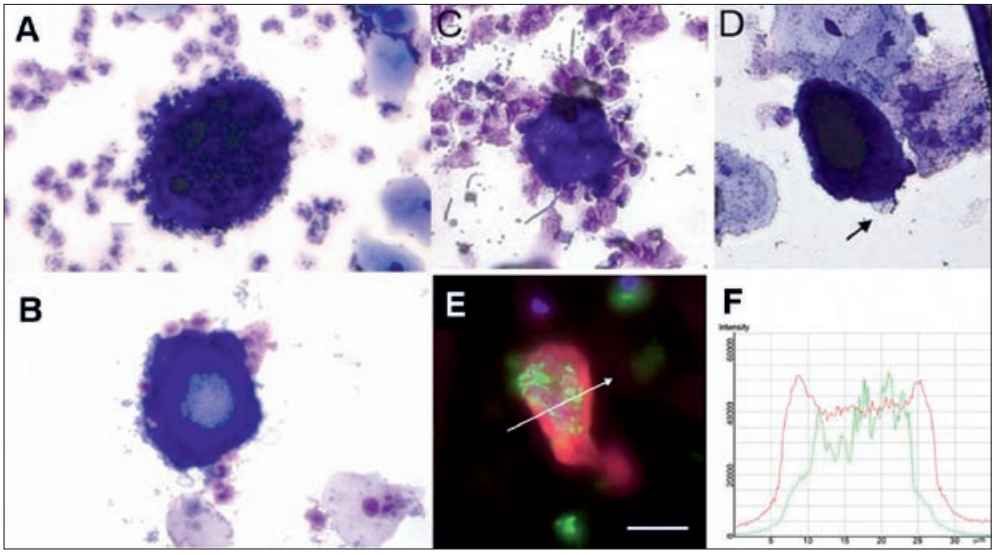

Fig. 3 (*A–B*) A human cystitis isolate was found to form IBCs within the mouse bladder, and IBCs were found in the stained urine sediment of the mouse (*A*) that were similar in morphology and size to those found in the original urine sediment from the human patient (*B*). (*C–D*) IBCs were found in the urine sediments of several patients with UPEC UTI. (*E–F*) Urine sediment IBCs were examined by immunofluorescence. A slice was taken through the middle of an IBC (*E*) and fluorescent intensity was analyzed along a traversing line (arrow). A representative fluorescent intensity distribution profile (*F*) shows peaks of uroplakin (red) staining corresponding to the facet membrane and *E. coli* (green) staining localized intracellularly (scale bar, 20 μm) (figure modified from Rosen et al. 2007).

from women with UTI and filamentous bacteria were found in 33 of 80 (41%) urines from women with UTI. None of the twenty urines from the asymptomatic control group showed evidence of IBCs or filaments. Furthermore, IBCs and filaments were never found in patients with UTI caused by Gram-positive pathogens, only in patients with UPEC infections, as IBCs are not a characteristic of experimental infection with Gram-positive uropathogens. Filamentous bacteria were highly associated with the presence of IBCs ($P < 0.001$). The presence of IBCs and filaments in the urines of women with acute UPEC infection suggests that the IBC pathogenic pathway characterized in the murine model also occurs in humans.

To further demonstrate this we infected mice with 18 different clinical UPEC strains from humans with UTI, and 15 of these strains were shown to proceed through the IBC cascade (Garofalo et al. 2007). Of the three strains that did not form IBCs, all were severely compromised in their ability to invade mouse urothelium as well. These findings suggest that this pathway is a common mechanism for establishment of UTI.

6. Modes of UPEC Persistence in the Urinary Bladder

UPEC invasion of the urothelial cells is critical not only for the establishment of acute infection through IBC formation, but also for chronic persistence within an intracellular reservoir (Mulvey et al. 1998, 2001, Schilling et al. 2002). Upon resolution of active infection and elimination of bacteriuria, we have found that UPEC remain within the murine urothelium inside LAMP1-positive vesicles (Fig. 4*A*) (Eto et al. 2006, Mysorekar and Hultgren 2006). These rosettes of typically 4–10 bacteria have ceased replicating and can remain viable for months in the murine host without eliciting an inflammatory response. Therefore, we have named this remaining UPEC population the quiescent intracellular reservoir (QIR). Upon epithelial turnover, these bacteria are capable of emerging to seed a new acute infection, and may represent a nidus for recurrence months after the initial infection.

In contrast, we have recently discovered that UPEC are also capable of persisting actively in the form of chronic cystitis (Hannan and Hultgren, unpublished data). While the hallmark of acute UPEC infection is the IBC, chronic bacterial cystitis in the mouse most closely resembles follicular cystitis in humans which is associated with prolonged UPEC bacteriuria and recurrent UTI (Hansson et al. 1990, Marsh et al. 1974). In the mouse, chronic cystitis is characterized by superficial colonization by UPEC of a dysplastic urothelium, marked epithelial reactivity and granulocyte infiltration, and the presence of large lymphoid nests or follicles within the bladder lamina propria (Figure 4*B–C*). The host and bacterial mechanisms that allow chronic cystitis to occur in the mouse are currently being investigated.

7. Conclusion

Our studies in a murine model of cystitis and translation of these findings to our understanding of human UTI are creating a new clinical paradigm with which to better understand the mucosal response to infectious disease and possible links to chronic inflammatory conditions. These findings will greatly facilitate the development of novel and improved strategies for the treatment and prevention of microbial infections.

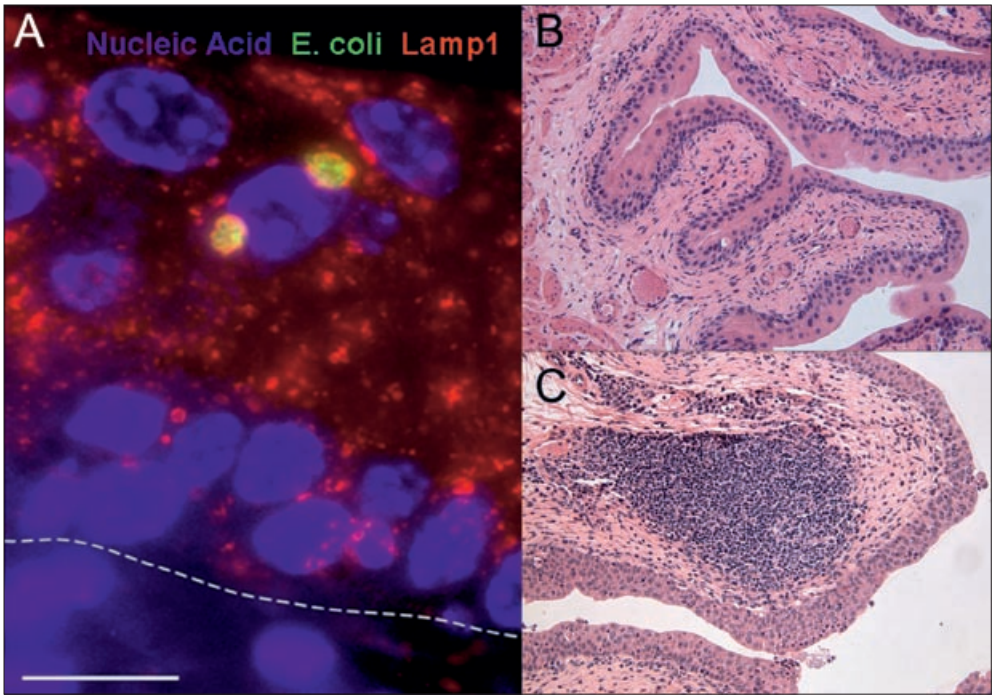

Fig. 4 Different outcomes of uropathogenic *E. coli* (UPEC) infection of the urinary tract. (*A*) Resolution of acute infection in C57BL/6J mice does not eliminate bacteria entirely from bladder tissue, as UPEC typically remain within quiescent intracellular reservoirs in LAMP1-positive vesicles inside the urothelial cells (scale bar, 10 µm; dashed line represents the urothelial basal lamina) (Mysorekar and Hultgren 2006). (*B–C*) Light micrographs of hematoxylin and eosin-stained sections of the mouse bladder wall 28 days post-intravesical infection of C3H/HeN mice with the human cystitis strain UTI89. UPEC infection may result either in resolution of acute cystitis and regeneration of normal urothelium (*B*), or the development of chronic follicular cystitis (*C*).

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Deconstruction of a Uropathogen – Another Route to Commensalism

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With 1 Figure

Abstract

It is generally accepted that bacterial pathogens can develop from commensal strains via acquisition of virulence-associated genes. In pathogenic *Escherichia coli* this often takes place via the acquisition of groups of genes located on plasmids and pathogenicity islands. We have studied the inverse phenomenon, namely the transition from pathogen to benign commensal-type organism. The prototypic asymptomatic bacteriuria strain 83 972 is capable of long-term colonization of the human bladder where it keeps a low profile *vis-à-vis* its human host. It does not trigger the usual countermeasures from the host. Many of these, for example exfoliation of bladder cells, cytokine production and influx of polymorphonuclear cells (PMNs), are activated by bacterial adhesins. Strain 83 972 has lost its three primary adhesins through adaptive mutations. However, it has adapted extremely well to its environmental niche. It is an excellent colonizer of the human urinary tract by virtue of its fast growth in urine and perhaps also its good biofilm formation characteristics. Indeed it performs so well that it can be used as a probiotic.

Zusammenfassung

Es ist allgemein akzeptiert, dass sich bakterielle Pathogene aus kommensalen Stämmen durch den Erwerb von Virulenz-assoziierten Genen entwickeln können. Bei pathogenen *Escherichia coli* geschieht dies häufig durch den Erwerb von Gengruppen, die auf Plasmiden oder Pathogenitätsinseln lokalisiert sind. Wir haben den umgekehrten Mechanismus untersucht, nämlich den Übergang von pathogenen in harmlose kommensale Organismen. Der prototypische Stamm 83 972, der von einem Patienten mit asymptomatischer Bakteriurie isoliert wurde, kann die Harnblase für lange Zeit kolonisieren, ohne im Wirt groß aufzufallen und die üblichen Gegenmaßnahmen im Wirtsorganismus auszulösen. Viele dieser Prozesse, wie z. B. die Ablösung von Blasenzellen, die Zytokinproduktion und das Einströmen von polymorphkernigen Leukozyten (PMNs), werden von bakteriellen Adhäsinen aktivitiert. Der Stamm 83 972 hat seine drei Hauptadhäsine durch adaptive Mutationen verloren. Er ist jedoch sehr gut an seine Umgebung angepasst. Durch sein schnelles Wachstum und vielleicht auch durch seine gute Biofilmproduktion ist dieser Stamm ein hervorragender Besiedler des menschlichen Urogenitaltraktes. Er ist in der Tat so effizient, dass er als Probiotikum eingesetzt werden kann.

Bacterial pathogens differ from commensals by expression of specific virulence factors. Commensals, in contrast, have traditionally been regarded as bacteria lacking such virulence factors or other specific mechanisms for interaction with host tissues. Asymptomatic carriage of bacteria may also result after a primary symptomatic infection. However, this type of carrier state differs from commensalism in that variants of pathogenic strains persist without evoking a host response. We have studied the molecular basis for attenuation of

virulence and adaptation to commensalism using the prototypic asymptomatic bacteriuria strain *Escherichia coli* 83972 as a model.

Urinary tract infections (UTIs) are among the most common infectious diseases of humans and a major cause of morbidity. It is estimated that 40–50 % of healthy adult women have experienced at least one UTI episode (Foxman 2002). Acute pyelonephritis and asymptomatic bacteriuria (ABU) represent the two extremes of UTI. *Escherichia coli* is responsible for more than 80 % of all UTIs. Acute pyelonephritis is a severe acute systemic infection caused by uropathogenic *E. coli* (UPEC) clones with virulence genes clustered on "pathogenicity islands". ABU, on the other hand, is an asymptomatic carrier state that resembles commensalism. Paradoxically, a large proportion of UTIs are caused by ABU *E. coli*. Individuals infected with ABU-group *E. coli* may carry high urine titers of a single *E. coli* strain for months or years without provoking a host response. According to conventional notion pathogens can develop from commensals by acquisition of virulence-associated genes located on, for example, pathogenicity islands or plasmids. The flip side of this evolutionary force is the equally important adaptation of the newly minted pathogen to its new host niche, where genes are inactivated either by point mutation, insertion, or deletion. We have found that the commensal-to-pathogen shift in UTI *E. coli* is bi-directional.

E. coli 83972 is a prototype ABU strain and was originally isolated from a young girl with ABU who had carried it for more than three years without symptoms. It is well adapted for growth in the urinary tract (UT) where it establishes long-term bacteriuria. The ability of UPEC to cause symptomatic UTI is enhanced by adhesins. The three primary fimbrial adhesive organelles associated with urovirulence are P, type 1 and F1C fimbriae. We investigated the genetic and molecular status of the three UPEC-class fimbriae in strain 83972 (Klemm et al. 2006, Roos et al. 2006a,b,c). It transpired that the strain is unable to express any of these adhesins in a functional form due to mutational events ranging from point mutations to major deletions.

Our observations support the notion that the ancestor of strain 83972 was a pyelonephritic UPEC strain. Indeed transcriptomics data suggest that the ancestor was very similar to the CFT073 prototype strain, a highly virulent pyelonephritis isolate that causes severe symptoms in the host (reviewed in Klemm et al. 2007). Strain 83972 has lost the capacity to express functional forms of many virulence factors but it still carries eroded versions of the corresponding genes. In humans strain 83972 failed to express virtually all known virulence-associated gene products (Roos and Klemm 2006). Due to this it does not trigger host defence mechanisms; however, it has kept a range of fitness factors such as efficient iron-acquisition systems.

Meanwhile, the lack of UPEC-class fimbriae in 83972 raises the issue of how it is able to persist in a high-flow environment like the human UT. Healthy adult humans normally produce ~2 liters of urine per day. An adult human bladder has a holding capacity volume of 200 to 400 ml, and micturition causes the release of roughly the same volume of urine; the volume of residual urine is about one milliliter. These would seem to be conditions that are hardly compatible with colonization by a non-adherent microbe. Meanwhile, strain 83972 grows extremely well in human urine *in vitro*, with a doubling time of less than 45 min (Roos et al. 2006a). Furthermore, we recently showed that the capacity to grow fast in urine is not a unique property of 83972, since several other ABU isolates also possess similar growth characteristics (Roos et al. 2006a). This suggests that fast growth in human urine to a large degree can account for the ability of *E. coli* 83972 to establish in the human bladder.

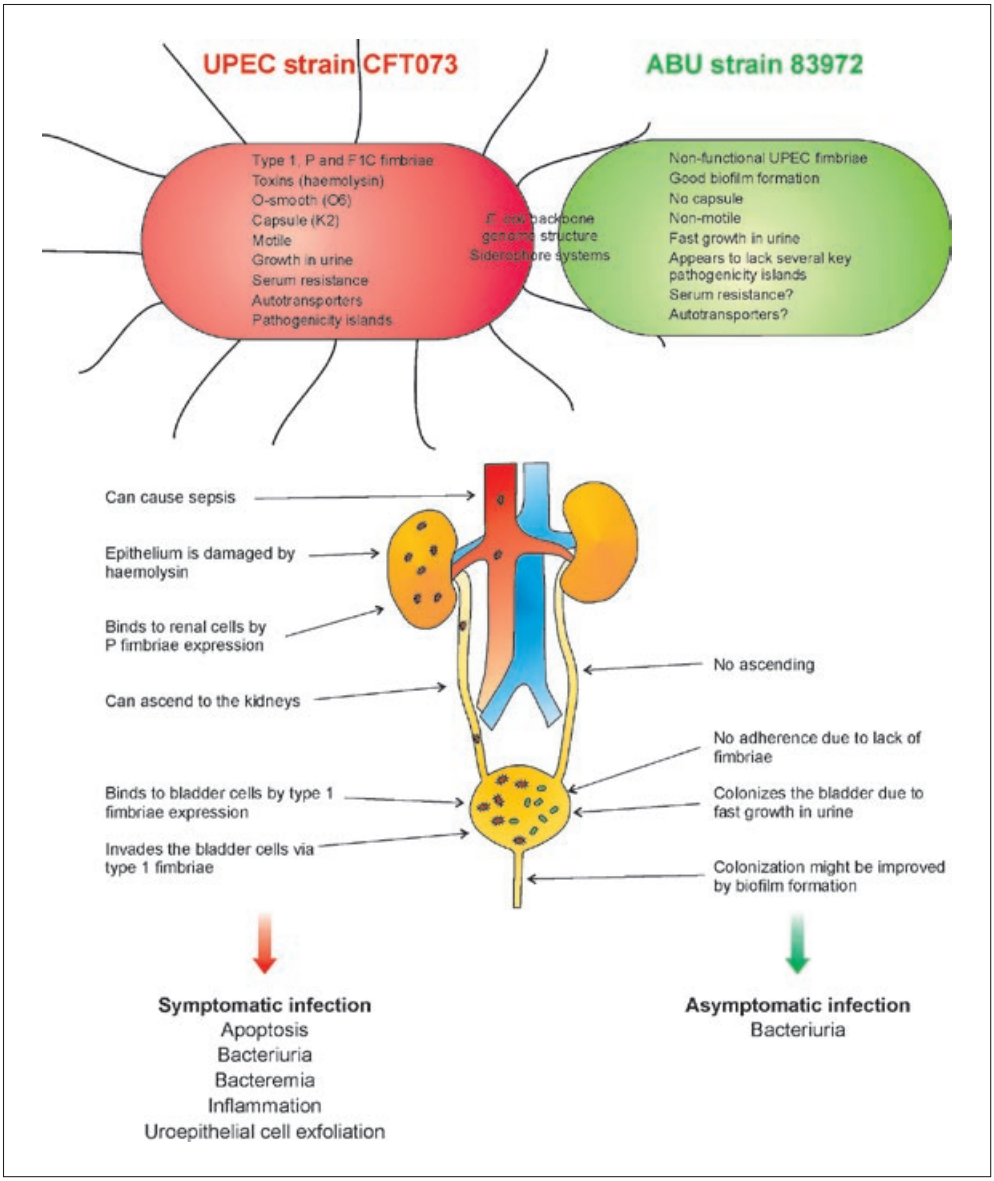

Fig. 1 Comparison of two prototypic UTI *E. coli*: the UPEC strain CFT073 and the ABU strain 83972

More than 50 % of all microbial infections have now been associated with the formation of biofilms. Biofilm formation is generally considered to be important for long-term bacterial establishment of surfaces submitted to hydrodynamic flow such as the human UT since bacterial biofilms are highly resistant to removal by liquid flow forces. We studied biofilm formation of strain 83 972 in human urine and found that it was an excellent biofilm former. In fact our data suggested that biofilm formation is a trait associated with

many ABU *E. coli* strains, but not with UPEC strains, and may be an important strategy for persistence in the urinary tract (Hancock et al. 2007, Hancock and Klemm 2007). In addition, global gene expression profiling of biofilm formation of 83972 in human urine revealed similarities with the expression profile in patients, indicating that biofilm formation might play a role for efficient colonization of the human UT (Hancock and Klemm 2007). Taken together, a picture of how ABU strain 83972 manages to colonize the human urinary tract is forming; the two primary strategies seem to be (*i*) fast growth and (*ii*) biofilm formation.

The long-term bacterial occupancy of a privileged host niche such as the human bladder must involve adaptations to the host environment. Strain 83972 had grown in the bladder of a girl for at least 3 years corresponding to more than 30,000 generations (Roos et al. 2006a). During this period of time the strain must have adapted considerably to this particular environmental niche. The strain lost the ability to express functional UPEC-class fimbriae probably as an evolutionary trade-off with the host defense. This ensured that it did not attract the attention of aggressive host defense mechanisms, such as cytokine production, inflammation and exfoliation of infected bladder cells. However, in order to avoid being flushed out of the system, it had to adapt to a particular ecological niche, i. e. human urine as growth medium and to optimize its growth rate to keep pace with the flow rate in the bladder. In line with this notion, strain 83 972 must have accrued genetic changes that have favored its fitness for growth in urine. Human urine is a very complex growth medium, and the composition of urine fluctuates daily. It is, however, known that iron availability is a limiting factor. Transcriptomics data indicated that strain 83 972 has adapted to growth in this iron-limiting environment by significantly increasing the expression of the majority of all known genes involved in iron uptake and transport (Roos and Klemm 2006, Hancock and Klemm 2007, Hancock et al. 2008). In fact it has adapted so well to its niche that it can out-compete a wide range of aggressive uropathogens.

Bacterial genomes are constantly changing, new genes are acquired by transfer and old ones are lost by mutations. It is thought that such changes in genetic repertoire are the primary mechanisms of bacterial adaptation to new environments. It is generally believed that commensal *E. coli* can become pathogenic through the acquisition of novel genetic information encoding virulence factors and niche-adaptation factors. Acquired genes range from single units to large constellations of genes such as pathogenicity islands or plasmids. Loss of genetic material has been observed to take place via mechanisms ranging from large-scale deletions to single point mutations. In contrast to organisms that have acquired genes for pathogenesis, *E. coli* 83 972 is an example of an organism that has adapted to commensalism through gene loss and mutations. The relationship between bacterium and host in a persistent infection is a mutual trade off. In this case the bacterium has lost its primary colonization factors; however, having done so it does not damage the host and evades immune surveillance. In effect the strain has become domesticated to a degree where it does not cause any symptoms: it has become benign.

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Ergebnisse des Leopoldina-Förderprogramms VI

Tagung und Berichte der Stipendiaten

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Deutschlands älteste Akademie, die Deutsche Akademie der Naturforscher Leopoldina, bemüht sich in besonderem Maße um die Förderung von Nachwuchswissenschaftlern. Seit 1992 vergibt sie zur Unterstützung der beruflichen Weiterentwicklung herausragender junger Wissenschaftlerinnen und Wissenschaftler ein Stipendium, ausgestattet durch Zuwendungen des Bundesministeriums für Bildung und Forschung, das es den Ausgezeichneten ermöglicht, innerhalb von zwei bis drei Jahren eigenständig ein außergewöhnlich innovatives Forschungsprojekt an ausländischen Wissenschaftseinrichtungen umzusetzen. Über 320 Forscherinnen und Forscher konnten seit Beginn des Programms gefördert werden. Der vorliegende Band zeigt die Vielfalt der Projekte und liefert Beispiele für die erreichten Ergebnisse seit 2006. Damit werden Chancen und Ansprüche des Förderprogramms für künftige Bewerber deutlich.

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Intestinal-Pathogenic *Escherichia coli*: Comparative Sequence Analysis, Differentiation by One-Step Multiplex PCR, and Identification of a Novel LEE-Associated Effector Protein

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With 1 Figure

Abstract

Intestinal pathogenic *Escherichia coli* represents a global health problem for mammals including humans. Intestinal disorders caused by the seven currently recognized pathotypes differ in the severity of clinical manifestations and prognosis. For EHEC, EPEC, and ATEC the pathogenicity island 'locus of enterocyte effacement' (LEE) encoding a type-III secretion system (T3SS) that injects various effector proteins directly into target cells determines the characteristic attaching-and-effacing (A/E) phenotype inducing pedestal formation on the surface of intestinal epithelial cells. To gain further insight into their interactions with eukaryotic target cells and to address a specific aspect of genome plasticity we analyzed the LEE of ATEC strains and compared their genetic organization and gene sequences with that of known LEE. We demonstrate that the core region of the LEE appears to be almost identical in all strains analyzed to date. However, the flanking regions of the various LEE exhibit large differences. Furthermore, we identified an additional *orf* present in many clinical EHEC, ATEC, and EPEC isolates encoding a new effector protein. To reliably identify and differentiate intestinal pathogenic *E. coli* a novel one-step multiplex PCR was developed. Interestingly, by using this MPCR we could identify unconventional intestinal pathogenic *E. coli* strains exhibiting mixed virulence factor profiles.

Zusammenfassung

Intestinal-pathogene *Escherichia coli* bilden ein weltweites Gesundheitsproblem für den Menschen und andere Säuger. Zurzeit sind sieben verschiedene Pathotypen bekannt, die unterschiedliche intestinale Erkrankungen verursachen können, die sich in ihrem Schweregrad, ihrer klinischen Manifestation und ihrer Prognose deutlich unterscheiden. Bei enterohämorraghischen *E. coli* (EHEC), enteropathogenen *E. coli* (EPEC) und atypischen EPEC (ATEC) bestimmt eine Pathogenitätsinsel, der „locus-of-enterocyte-effacement (LEE)", den charakteristischen A/E-Phänotyp (A/E: „attaching-and-effacing"), der durch die Bildung sockelartiger, aktinreicher Strukturen auf der Oberfläche infizierter intestinaler Epithelzellen gekennzeichnet ist. Die LEE-Pathogenitätsinsel kodiert für ein Typ-III-Sekretionssystem (T3SS), durch das Effektorproteine direkt in die Zielzelle injiziert werden können. Um weitere Einblicke in die Interaktionen mit eukaryotischen Zielzellen zu gewinnen und um spezifische Aspekte der Genomplastizität zu beleuchten, haben wir die genetische Organisation und die Gensequenzen der LEE-PAI von ATEC-Stämmen mit den Sequenzen bereits bekannter LEE verglichen. Die Kernregion des LEE scheint in allen bisher bekannten Stämmen nahezu identisch zu sein, während die flankierenden Bereiche große Unterschiede aufweisen. Zusätzlich konnten wir einen weiteren offenen Leserahmen identifizieren, der in vielen klinischen EHEC-, ATEC- und EPEC-Isolaten vorliegt und für ein neues T3SS-Effektorpro-

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tein kodiert. Zum sicheren Nachweis und zur Differenzierung von intestinal-pathogenen *E. coli* wurde eine neue „one-step multiplex PCR (MPCR)“ entwickelt. Interessanterweise konnten wir mit dieser MPCR auch untypische intestinal-pathogene *E. coli-Stämme* mit einem gemischten Virulenzfaktorprofil identifizieren.

1. State of the Art

Although being regarded commonly as a beneficial inhabitant of the gastrointestinal tract, distinct isolates of *Escherichia coli* exhibit a high pathogenic potential. Currently, diarrheagenic *E. coli* bacteria can be grouped into seven pathotypes: enteropathogenic *E. coli* (EPEC), atypical EPEC (ATEC), 'locus of enterocyte effacement (LEE)'-positive and LEE-negative Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC), and enteroaggregative *E. coli* (EAEC). EPEC and ATEC are major etiological agents of infant diarrhoea predominantly in developing countries (Kaper et al. 2004, Ahmed et al. 2008). Infections with Shiga toxin-producing *E. coli* (STEC) can lead to various outcomes ranging from asymptomatic carriage and uncomplicated diarrhoea to bloody diarrhoea and the hemolytic uremic syndrome (HUS) – a leading cause of acute kidney failure in young children (Tarr et al. 2005). EPEC, ATEC, and LEE-positive STEC elicit the attaching-and-effacing (A/E) phenotype that is characterized by the degeneration of microvilli followed by an intimate attachment of bacteria to the target cell membrane. The A/E phenotype as well as virulence factors essential for full pathogenicity are associated with the presence of the 35-kb LEE pathogenicity island. Diarrheagenic *E. coli* strains exhibit characteristic virulence factor profiles that have been widely used for pathotyping. Routine diagnostic procedures are currently based on combinations of biochemical tests, serotyping, phenotypic assays based on known virulence characteristics, and are further supported by methods employing molecular biology techniques (Ahmed et al. 2008).

In this study we have analyzed several atypical enteropathogenic *E. coli* (ATEC) isolates by comparative sequence analysis of the LEE, employed these analyses to develop a novel one-step multiplex PCR, and, furthermore, identified a new LEE-associated *orf* encoding a novel T3SS-dependent effector protein.

2. Comparative Sequence Analyses of LEE Pathogenicity Islands

LEE described thus far are chromosomally integrated at one of three tRNA loci (*selC*, *pheU*, *pheV*). To analyze LEE pathogenicity islands in further detail, we sequenced the LEE of ATEC strains B6 (*pheU*, O26) and 9812 (*pheV*, O128) and compared these sequences with LEE sequences reported in the data-base. The sequence of LEE-B6 is integrated at the *pheU* locus and is comparable to the LEE of strains RDEC-1, 83/39 and 413/89–1 containing IS sequences at both sides of the core region and the *lifA/efa1* region in the 3′ flanking region. The sequence of LEE-9812 is integrated in the *pheV* region and exhibits a mosaic organization consisting of gene are homologues to elements of prophage CP4-44, an IS3 element, the LEE core region, followed by sequences that are homologous to elements on the LEE-PAI of either BSTEC RW1374 and REPEC 84/110-1 or STEC O157:H7. Detailed comparative sequence alignments of the core regions showed that the LEE of nearly all analyzed strains – including the newly sequenced LEE of ATEC strain B6 and ATEC strain 9812 – exhibit the same genes in identical order

and orientation as the reference strain E2348/69. In contrast, the flanking regions of the LEE-PAI of ATEC B6 and ATEC 9812 harbor different additional sequences. The core regions of the LEE of 9812 and B6 were found to be highly homologous to each other (on average 99.5 % identity on DNA level) and to the LEE of the strains REPEC RDEC-1 (98.8 %), REPEC 83/39 (98.8 %), BSTEC 413/89-1 (99.4 %) and BSTEC RW1374 (96.9 %). In contrast to the core regions with highly homologous genes and also sizes (34.4 kb ± 0.7 kb, 38.4 % GC ± 0.2 %), the overall sequences of LEE core and flanking regions show higher variability (between 35.6 kb [E2348/69] and 111.0 kb [RW1374], between 38.4 % [E2348/69] and 45.0 % [RW1374] GC % content). The lower GC % content of the LEE core region of about 38.4 % in comparison to the GC % content of the total *E. coli* genome of about 50.8 % strongly emphasizes the LEE to be derived from a foreign ancestor by horizontal gene transfer. Moreover, the higher GC % contents of the flanking regions support a subsequent remodeling of the flanking regions through possibly multiple recombination events. In addition, our results indicate that – at least with respect to the LEE – ATEC strains do not represent a separate phylogenetic lineage but that they can be arranged with LEE of other strains in distinct groups. This also indicates that the varying pathogenicity of ATEC isolates should be modulated by factors not associated with the LEE. Further details of these analyses will be published elsewhere (Müller et al. 2008, to be submitted).

3. Identification of a Novel LEE-Associated Effector Protein (Ibe)

Upon sequencing the flanking regions of various ATEC strains we identified a novel *orf* designated *rorf0* encoding a 32 kDa protein which was named Ibe for 'IQGAP1-binding effector'. Interestingly, *rorf0* was not found in the EPEC prototype strain E2348/69. A survey of a collection of LEE-harboring A/E strains by specific PCR for *rorf0* showed that *rorf0* is most frequently found in EHEC strains (~ 80 %) followed by ATEC strains, and is less frequently harbored in EPEC isolates (~ 16 %). Ibe is a T3SS-dependent secreted protein which is translocated to the cytosol of the target host cell where it accumulates at the sites of pedestal formation. Pull-down experiments show that Ibe interacts with a host cell protein that was identified as the scaffolding protein IQGAP1 that is involved in the regulation of cell morphology and motility. Therefore, we conclude that Ibe might be involved in processes leading to the rearrangement of the actin cytoskeleton. These findings are further supported by immunofluorescence analysis demonstrating the co-localization of Ibe with IQGAP1 at the site of the pedestals. Further characterization of the Ibe protein, its distribution among intestinal pathogenic *E. coli*, and its role in the modulation of target cell responses are ongoing and will be reported elsewhere (Buss et al. in preparation).

4. Novel One-Step Multiplex PCR (MPCR)

Based on the comparative sequence analyses briefly described above we developed a one-step MPCR for the simultaneous identification of the seven currently established pathotypes of intestinal pathogenic *Escherichia coli*: enteropathogenic (EPEC), atypical enteropathogenic (ATEC), enterohemorrhagic (EHEC), Shiga-toxin-encoding (STEC; LEE-positive and LEE-negative), enteroinvasive (EIEC), enterotoxigenic (ETEC), and enteroaggrega-

tive (EAEC) *E. coli*. The MPCR incorporates 12 primer pairs that have been optimized for MPCR-compatible properties including non-interference and the generation of DNA fragments to be easily resolved by standard agarose gel electrophoresis (MÜLLER et al. 2006, 2007). To cover all known pathotypes we designed primer pairs for the *escV*, *bfpB*, *stx1*, *stx2*, *elt*, *estIa*, *estIb*, *invE*, *astA*, *aggR*, *pic*, and *ent* genes, as well as the *uidA* gene as a general marker for *E. coli* (ca. 98 % coverage). Each primer pair was selected to represent a gene-specific consensus sequence derived from the accessible information for each gene in the data-base. The MPCR was validated by using a set of reference strains for each pathotype from our collection. All specific pathotypes were easily detected in a single reaction and no cross-priming could be observed (Fig. 1). The identities of the products obtained by MPCR were verified by nucleotide sequence analysis for the respective reference strains employed.

Marker gene	Product size [bp]	C600	EPEC	ATEC	LEE$^{pos.}$ STEC	LEE$^{neg.}$ STEC	ETEC	EIEC	EAEC
uidA	1487	+	+	+	+	+	+	+	+
escV	544	-	+	+	+	-	-	-	-
bfpB	910	-	+	-	-	-	-	-	-
stx1 / stx2	244 / 324	-	-	-	+	+	-	-	-
elt	655	-	-	-	-	-	+	-	-
estIa / estIb	157 / 171	-	-	-	-	-	+	-	-
invE	766	-	-	-	-	-	-	+	-
astA	102	-	+/-	+/-	+/-	+/-	+/-	+/-	+
aggR	400	-	-	-	-	-	-	-	+
pic	1111	-	-	-	-	-	-	+/-	+

Fig. 1 Schematic representation of amplified DNA fragments to be obtained by the novel multiplex PCR (MPCR). The matrix of amplicons allows for a reliable classification of the particular pathotype of the isolate.

The MPCR was applied to the analysis of clinical isolates in our strain collection that had been obtained from several geographic regions. All strains were identified by the MPCR to exhibit either a specific pattern of virulence genes and, therefore, to belong to one of the known pathotypes, or to harbor none of the tested virulence-associated genes. However, there were a few exceptions exhibiting virulence gene patterns that allowed no association with any of the accepted pathotypes but expressed rather unconventional mixed virulence factor patterns such as e. g. the presence of the EAF plasmid expressing BFP pili without harboring the LEE. Further characterization of their particular phenotypes by complementary approaches including PCR analysis, cytotoxicity assays, immunofluorescence, monitoring of cell adhesion etc. verified the results obtained by MPCR and clearly demonstrated that these strains cannot be assigned unambiguously to a specific pathotype. Hence, these strains were designated intermediate strains (MÜLLER et al. 2007).

5. Conclusions

In recent years the astonishing plasticity of the *E. coli* genome has been well recognized and various attempts have been made to identify and describe putative phylogenetic lin-

eages and the mechanisms of gene transfer involved (Ahmed et al. 2008, Reyes-Lamothe et al. 2008). The recognition of substantial genome plasticity should also have an impact on strategies to identify and differentiate intestinal pathogenic isolates. Hence, we investigated clinical intestinal pathogenic *E. coli* isolates from different geographic regions with a special emphasis on the LEE-harboring pathotypes. Sequence comparisons also demonstrated that the core genes of the LEE are highly homologous whereas the gene content of the flanking regions appears to reflect the individual history of the particular strain. Comparative analysis of various LEE resulted in the identification and partial characterization of a novel T3SS-dependent effector protein (Ibe) that was found in many EHEC and ATEC strains but is missing in the EPEC prototype strain E2348/69. Ibe interacts with IQGAP1 and appears to be involved in actin rearrangements during pedestal formation. Based on our analyses we designed a novel one-step MPCR allowing the identification and differentiation of all known intestinal pathotypes of *E. coli*. Applying this MPCR we identified intermediate *E. coli* strains that exhibit a mixed repertoire of virulence factors which does not fit into any one accepted pathotype. These finding further reflect the enormous plasticity of the *E. coli* genome (Bielaszewska et al. 2007a,b). In addition, this study clearly emphasizes the fact that index virulence factors are not restricted to one particular pathogroup but that they can occur also in other pathotypes where they might contribute to their strain-specific virulence potential. This also implies that the classification of a particular isolate should be supported by a genetic profile as large as possible. In a practical setting the newly developed MPCR would certainly provide a means that is to do so.

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Adherent-Invasive *Escherichia coli* and Colonization of the Ileal Mucosa in Crohn's Disease Patients

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Abstract

Abundant data have incriminated intestinal bacteria in the initiation and amplification stages of the two major inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC). Increased numbers of mucosa-associated *E. coli* are observed in both CD and UC and the association of CD with invasive *E. coli* was reported by several groups. Based on their pathogenicity features, CD-associated *E. coli* were termed adherent-invasive *E. coli* (AIEC). AIEC strains are able to invade intestinal epithelial cells and to replicate extensively within macrophages inducing the secretion of large amounts of TNF-α. They are isolated from ileal biopsies of 36.4 % of patients with ileal involvement of CD, and their presence is due to abnormal ileal expression of CEACAM6, which is a receptor for type 1 pili.

Zusammenfassung

Zahlreiche Daten belegen, dass intestinale Bakterien an der Auslösung und Ausdehnung der beiden wichtigsten chronisch-entzündlichen Darmerkrankungen, dem Morbus Crohn (CD) und der ulcerativen Colitis (UC), beteiligt sind. Ein Anstieg der mit der Mukosa assoziierten *E. coli*-Bakterien konnte sowohl bei CD als auch UC beobachtet werden. Mehrere Arbeitsgruppen berichteten von einem Zusammenhang zwischen CD und invasiven *E. coli*. Aufgrund ihrer pathogenen Eigenschaften wurden mit CD assoziierte *E. coli* auch als adhärent-invasive *E. coli* (AIEC) bezeichnet. AIEC-Stämme sind in der Lage, in intestinale Epithelzellen einzudringen und sich effektiv in Makrophagen zu vermehren. Dabei lösen sie die Sekretion größerer Mengen von TNF-α aus. Sie werden aus den ilealen Biopsien von 36,4 % der Morbus-Crohn-Patienten isoliert, bei denen das Ileum betroffen ist. Die Anwesenheit der AIEC-Bakterien kann auf eine veränderte ileale Expression von CEACAM6 zurückgeführt werden. CEACAM6 ist ein Rezeptor für Typ-1-Fimbrien.

Crohn's disease and ulcerative colitis are chronic disorders of the gastrointestinal tract with a combined prevalence of about 150–200 cases per 100,000 in Western countries. Despite decades of research, the etiology of CD remains unknown. Its pathogenesis involves a complex interplay between host genetic, immune dysfunction and microbial or environmental factors. Several lines of evidence implicate microorganisms of the intestinal microbiota in the pathogenesis of CD. Improvement in clinical disease occurs in CD patients receiving prolonged courses of antibiotics (Sartor 2004). In addition, exposure of the terminal ileum post-surgically to luminal contents is associated with increased inflammation, and diversion of the faecal stream is associated with improvement (Rutgeerts et al. 1991). Generalized or localized dysbiosis is observed in IBD, corresponding to the presence of low numbers

of usual bacteria, high numbers of unusual bacteria and sometimes a reduction in biodiversity. Studies of luminal bacterial composition in patients with IBD using culture and molecular techniques have reported a decrease in beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* species and an increase in pathogenic bacteria such as *Bacteroides* and *Escherichia coli* (Swidsinski et al. 2002, Neut et al. 2002). Such dysbiosis induces a breakdown in the balance between putative species of "protective" versus "harmful" intestinal bacteria and may promote inflammation.

The search for infectious agents likely to cause CD has focused mainly on intracellular pathogens. Indeed, with the identification of mutations in the NOD2-encoding gene in patients with CD and given the intracellular location of NOD2, the presence of pathogenic invasive bacteria could be the link between innate immune response to invasive bacteria and the development of the inflammation (Hugot et al. 2001, Ogura et al. 2001). Aphthous ulcer, a necrosis of M-cells of Peyer's lymphoid follicles, is recognized as the earliest lesion of CD. Such ulcers occur in shigellosis, salmonellosis and yersinial enterocolitis, in which invasiveness is an essential virulence factor of the bacteria involved in the disease. This supports also a putative role for invasive pathogen(s) in the initiation of CD lesions. Three independent studies have reported the presence of intramucosal *E. coli* in IBD patients or mucosa-associated *E. coli* with invasive properties (Darfeuille-Michaud et al. 2004, Martin et al. 2004, Sasaki et al. 2007). The invasive process of CD-associated *E. coli* strains is dependent upon both functioning host cell actin microfilaments and microtubules (Boudeau et al. 1999). Electron microscopic examination of epithelial cells infected with CD-associated *E. coli* LF82 revealed a macropinocytosis-like process of entry, characterized by elongation of the membrane extensions that surrounded the bacteria at the sites of contact between the entering bacteria and the epithelial cells. After internalization, bacteria lyse the endocytic vacuole and replicate in the host cell cytoplasm. The invasive process of CD-associated *E. coli* strain LF82 is unique since it does not possess any of the known genetic invasive determinants described for enteroinvasive, enteropathogenic, and enterotoxigenic *E. coli,* and *Shigella* strains. The virulence factors that play a role in the invasive ability of strain LF82 are type 1 pili inducing membrane extension (Boudeau et al. 2001), flagella conferring bacteria motility and regulating type 1 pili expression (Barnich et al. 2003, Claret et al. 2007), outer membrane vesicles delivering bacterial effectors to host cells (Rolhion et al. 2005) and the outer membrane protein OmpC, which regulates the expression of most virulence factors (Rolhion et al. 2007).

The behavior of the CD-associated invasive *E. coli* within macrophages is different from that of other invasive bacteria. Invasive *E. coli* strains isolated from CD patients are able to survive and to replicate extensively within murine macrophages in a large vacuole (Glasser et al. 2001). However, whereas most invasive bacteria induce cell death of infected macrophages, no necrosis or apoptosis of macrophages infected with CD-associated invasive *E. coli* was observed even after 24 h post-infection. Moreover, in contrast to many pathogens that escape from the normal endocytic pathway or infiltrate autophagy, CD-associated invasive *E. coli* are taken up by macrophages within phagosomes, which mature without diverting from the classical endocytic pathway, and which share features with phagolysosomes (Bringer et al. 2006). To survive and replicate in the harsh environment encountered inside these compartments, including acid pH and proteolytic activity of cathepsin D, bacteria have elaborated adaptation mechanisms in which acidity constitutes a key signal for activating the expression of virulence genes (Bringer et al. 2005). Macrophages infected with CD-as-

sociated invasive *E. coli* release large amounts of tumor necrosis factor α (TNF-α) (Glasser et al. 2001). Continuous macrophage activation and TNF-α release are due to the sustained multiplication of intracellular bacteria within the phagosomes.

On the basis of the pathogenic traits of CD-associated *E. coli*, a new potentially pathogenic group of *E. coli* was designated AIEC for Adherent-Invasive *Escherichia coli*. The criteria for inclusion in the group are:

(*i*) ability to adhere to and to invade intestinal epithelial cells with a macropinocytosis-like process of entry dependent on actin microfilaments and microtubules recruitment,
(*ii*) ability to survive and to replicate extensively in large vacuoles within macrophages without triggering host cell death, and
(*iii*) ability to induce the release of large amounts of TNF-α by infected macrophages.

Adherent-invasive *E. coli* strains were found to be highly associated with ileal mucosa in CD patients (Darfeuille-Michaud et al. 2004, Sasaki et al. 2007). AIEC strains are preferentially found in early recurrent CD lesions after surgery, thus pleading for a possible role in the initiation of inflammation and not only as secondary invaders.

The high prevalence of AIEC strains associated with the ileal mucosa observed in CD patients suggested an abnormal expression and/or tissue tropism of a specific host receptor recognized by bacterial lectin-like surface adhesins in a genetically predisposed host gut segment. CD-associated AIEC strains adhere to the brush border of primary ileal enterocytes isolated from CD patients but not from controls without inflammatory bowel disease (Barnich et al. 2007). Most AIEC strains associated with CD ileal mucosa express a type 1 pili variant. Expression of type 1 pili variant, compared to that of K-12 type 1 pili, enhanced bacterial adhesion to the brush border of primary ileal enterocytes from CD patients. Adhesion inhibition experiments in the presence of antibodies raised against the putative type 1 pili receptors indicated that AIEC strains bind to the carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) expressed on the apical side of ileal epithelial cells.

CEACAM6 acting as a receptor for AIEC adhesion is abnormally expressed by ileal epithelial cells in CD patients and its expression was observed in both uninvolved and inflamed ileal mucosa. Increased CEACAM6 expression was observed in cultured intestinal epithelial cells after interferon-gamma or TNF-alpha stimulation and after infection with AIEC bacteria, indicating that AIEC can promote their own colonization in CD patients. Thus, the presence of AIEC bacteria and the secretion of pro-inflammatory cytokines lead to an amplification loop of colonization and inflammation.

The etiopathogenesis of ileal CD seems to be intimately linked to the presence of Adherent-Invasive *E. coli*. The high prevalence of AIEC in patients with ileal involvement of CD could be a first step in the establishment of a modified Koch's postulate, which needs to take into account the genetic susceptibility of the host. The interaction between AIEC and the host receptor CEACAM6 is a novel example of a bacterial pathogen subverting functional membrane-bound proteins as receptors for colonizing epithelia.

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Die Gründung der Leopoldina – *Academia Naturae Curiosorum* – im historischen Kontext

Johann Laurentius Bausch zum 400. Geburtstag

Leopoldina-Symposium
vom 29. September bis 1. Oktober 2005 in Schweinfurt (Bibliothek Otto Schäfer)

Acta Historica Leopoldina Nr. 49
Herausgegeben von Richard Toellner (Kloster Amelungsborn), Uwe Müller (Schweinfurt), Benno Parthier und Wieland Berg (Halle/Saale)
(2008, 336 Seiten, 42 Abbildungen, 22,95 Euro, ISBN 978-3-8047-2471-6)

Ziel dieser interdisziplinären, internationalen Tagung war es, die Gestalt des Johann Laurentius Bausch (1605–1665) in ihren biographischen, sozialen und wissenschaftsgeschichtlichen Bedingungen darzustellen sowie die Gründung der Leopoldina in den Rahmen der internationalen Akademiengeschichte des 17. Jahrhunderts einzuordnen. Es wurde der über die bisherige Literatur hinausgehende aktuelle Forschungsstand in neun Vorträgen präsentiert, die der vorliegende Band in erweiterter und aktualisierter Form dokumentiert und vertieft durch Anhänge mit der Edition der *Leges* der Akademie und Bibliographien der im frühen Akademieprogramm veröffentlichten Monographien und ihrer Vorgänger aus anderthalb Jahrhunderten sowie einer Analyse der Selbstdarstellung der Leopoldina in ihrer Korrespondenz mit der Royal Society von 1670 bis 1677.

Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

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The Adhesion and Invasion of Crohn's Disease-Associated *Escherichia coli* is Controlled by the Flagellar Sigma Factor FliA and a c-di-GMP-Dependent Pathway

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Abstract

The invasion of intestinal epithelial cells by the adherent-invasive *Escherichia coli* (AIEC) strain LF82 depends on type 1 pili. The absence of flagella in the LF82-Δ*flhD* and LF82-Δ*fliA* mutants, lacking $FlhD_2C_2$ and FliA flagellar regulators, induces a decrease of adhesion and invasion abilities and type 1 pili synthesis. FliA expressed alone was sufficient to restore these defects in both mutants by inducing the *yhjH* gene encoding the YhjH protein, a phosphodiesterase involved in the degradation of the bacterial second messenger c-di-GMP. Deletion of the diguanylate cyclase YaiC involved in c-di-GMP synthesis also produced partial restoration of these defects. These results show that in the strain LF82, FliA sigma factor is a regulatory component linking flagellar and type 1 pili synthesis, via the c-di-GMP-dependent pathway.

Zusammenfassung

Die Invasion intestinaler Epithelzellen durch den adhäsiv-invasiven *Escherichia coli* (AIEC) Stamm LF82 hängt von Typ-1-Fimbrien ab. Das Fehlen der Flagellen und der Flagellenregulatoren $FlhD_2C_2$ und FliA in LF82-Δ*flhD* und LF82-Δ*fliA* Mutanten führt zu einer Verminderung der Adhäsions- und Invasionsfähigkeit sowie der Typ-1-Fimbriensynthese. Die Expression von FliA allein genügt, um diese Defekte in beiden Mutanten aufzuheben. Dadurch wird die Expression des *yhjH*-Gens induziert, welches das YhjH-Protein kodiert. Die Phosphodiesterase YhjH ist am Abbau des bakteriellen sekundären Botenstoffes c-di-GMP beteiligt. Auch die Deletion der Diguanylat-Zyklase YaiC, die an der c-di-GMP-Synthese beteiligt ist, führte zu einer teilweisen Wiederherstellung der Defekte. Diese Ergebnisse zeigen, dass der FliA-Sigmafaktor im Stamm LF82 eine regulatorische Funktion hat und über den c-di-GMP-abhängigen Signalweg die Flagellen- und Typ-1-Fimbriensynthese vernetzt.

Our study concerns a pathogenic group of *E. coli* strains associated with ileal lesions of Crohn's disease (CD) (Darfeuille-Michaud et al. 2004). The strains are able to adhere to and to invade intestinal epithelial cells and are named adherent-invasive *E. coli* (AIEC) (Boudeau et al. 1999). AIEC adhesion and invasion depend on the type 1 pili that induce membrane extensions of epithelial cells (Boudeau et al. 2001). However, the type 1 pili of AIEC reference strain LF82 are not able to confer invasiveness to a nonpathogenic *E. coli* strain K-12, indicating that the genetic background of AIEC is essential. Flagella also play important roles in the adhesion and invasion of strain LF82 (Barnich et al. 2003). The afla-

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gellar LF82-Δ*fliC* mutant shows a drastic downregulation of type 1 pili synthesis, a decrease in adhesion and invasion abilities and a decrease in the flagellar regulator *flhDC* mRNA levels indicating that in strain LF82 flagellar motility and other virulence factors are coregulated.

In order to study the role of flagellar regulators $FlhD_2C_2$ and FliA in the ability of AIEC strain LF82 to adhere to and to invade Intestine-407 epithelial cells, the LF82-Δ*flhD* and LF82-Δ*fliA* deletion mutants were constructed. The adhesion and invasion levels of LF82-Δ*flhD* and LF82-Δ*fliA* mutants were significantly lower than those of strain LF82. Thus, in strain LF82, the $FlhD_2C_2$ and FliA regulators play key roles to allow bacteria to enter intestinal epithelial cells.

A decreased ability to adhere to and invade epithelial cells has already been shown to depend on lower type 1 pili levels in strain LF82 (Boudeau et al. 2001, Barnich et al. 2003). We therefore measured expression of type 1 pili in the LF82-Δ*flhD* and LF82-Δ*fliA* mutants by monitoring bacterial aggregation of yeast cells. Both mutants were strongly impaired in their ability to aggregate yeast cells compared to the wild type strain. The deficiency in type 1 pili is a consequence of the shift of the DNA invertible element orientation toward the phase-OFF orientation. This suggests that, in strain LF82, $FlhD_2C_2$ and FliA are involved in the regulation of type 1 pili through the transcriptional control of the *fim* operon. The expression levels of $FlhD_2C_2$ depend, in the flagellar gene hierarchy, on the presence of FliA and *vice versa*. Adhesion and invasion of LF82-Δ*flhD* and LF82-Δ*fliA* were fully complemented with pBAD*flhDC* and pBAD*fliA*, respectively. In addition, the overexpressed *fliA* gene specifically and fully restored the defects of the LF82-Δ*flhD* mutant. Thus, the full invasiveness of AIEC strain LF82 depends on the FliA sigma factor.

To identify mediators of FliA action on the synthesis of type 1 pili in strain LF82, we studied the involvement of the *yhjH* gene which is controlled by FliA in *E. coli* K12 and *Salmonella* Typhimurium strains (Ko and Park 2000, Frye et al. 2006). The *yhjH* gene encodes the YhjH protein carrying an EAL domain which functions as a phosphodiesterase for the second messenger c-di-GMP (Ryjenkov et al. 2006) and stimulates flagellar motility in *E. coli* K-12 (Ko and Park 2000). Interestingly, the transformation of LF82-Δ*fliA* mutant with pBAD*yhjH* partially restored adhesion and invasion levels. This restoration was due to increased type 1 pili synthesis. In strain LF82, it exists also a regulation of *yhjH* gene expression by FliA. The levels of *yhjH* mRNA were up to 100-fold higher in LF82 than in LF82-Δ*fliA*. These results suggest that in strain LF82, FliA regulates type 1 pili, adhesion and invasion by acting via the YhjH expression. The c-di-GMP turnover is involved in the ability of strain LF82 to adhere to and to invade intestinal epithelial cells. Indeed, in the genetic background of LF82-Δ*fliA*, a deletion of *yaiC,* which encodes a diguanylate cyclase involved in c-di-GMP synthesis (Simm et al. 2004), resulted in increased adhesion and invasion levels.

A regulatory pathway linking flagella and type 1 pili synthesis exists in the CD-associated AIEC strain LF82 and is involved in the ability of this strain to adhere to and to invade intestinal epithelial cells. The FliA-dependent *yhjH* gene, encoding the c-di-GMP phosphodiesterase YhjH required for the breakdown of the novel second messenger c-di-GMP, is a mediator linking FliA to the regulation of virulence determinants. The role of c-di-GMP in the control of type 1 pili expression, adhesion and invasion abilities of strain LF82 fits with the concept that lower c-di-GMP levels promote virulence. This is a novel model for regulation of type 1 pili synthesis involving the c-di-GMP-dependent pathway (Claret et al. 2007).

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Vorträge und Abhandlungen zur Wissenschaftsgeschichte 2002/2003 & 2003/2004

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Der Band enthält elf Vorträge aus den Wissenschaftshistorischen Seminaren der Leopoldina vom Wintersemester 2002 bis Sommersemester 2004: Die Rekonstruktion der angeblichen Verwicklung Adolf Butenandts in die NS-Forschung (Achim Trunk, Köln); die „Aufforderung" Wilhelm Wiens an die deutschen Physiker am Beginn des Ersten Weltkrieges (Stefan L. Wolff, München); zum Naturbegriff in der mittelalterlichen Medizin (Ortrun Riha, Leipzig); Raphael Lemkins Standpunkt über Menschenversuche und Genozid im Nürnberger Ärzteprozess 1946/47 (Paul Weindling, Oxford, UK); die Rassenhygienikerin Agnes Blum und die Frauenbewegung (Johanna Bleker, Berlin); die Vorgeschichte der Entdeckung der DNA-Doppelhelix und die Rolle des Protein-Paradigmas (Rudolf Hagemann, Halle/Saale); Politik und Mathematik während der „Kulturrevolution" in China (Joseph W. Dauben, New York); Eugenik und Humangenetik am Beispiel der psychiatrischen Genetik in Deutschland, Großbritannien und den USA zwischen 1910 und 1960 (Volker Roelcke, Gießen); Karl Ziegler und 50 Jahre Niederdruck-Polyethylen (Horst Remane, Halle/Saale); Rudolf Virchows Strategie des Sammelns am Beispiel seines Pathologischen Museums (Thomas Schnalke, Berlin); Natur als Erklärungshilfe in den Bilderhandschriften des Sachsenspiegels (Heiner Lück, Halle/Saale).
Als Abhandlungen folgen wissenschaftshistorische Analysen zur Erklärung des Regenbogens bei Johann Wolfgang von Goethe und Josef Maria Pernter (Thomas Nickol, Halle/Saale) sowie zur Zusammenarbeit von Werner Reichardt und Bernhard Hassenstein auf dem Weg zur Tübinger Biokybernetik (Bernhard Hassenstein, Merzhausen).
Abgeschlossen wird der Band durch zwei Dokumentationen (Uwe Müller, Schweinfurt, dokumentiert Alexander von Humboldts Mitwirkung an der Aufnahme Aimé Bonplands in die Akademie und ediert das dazu vorhandene Archivgut, und Rudolf Hagemann, Halle/Saale, beschreibt die Entwicklung der Genetik an der Universität Halle bis zum Ende des 20. Jahrhunderts, hier als Teil II für die Zeit ab 1946).

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Molecular and Evolutionary Mechanisms Underlying the Emergence of Non-O157:H7 Enterohaemorrhagic *Escherichia coli*

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With 1 Figure and 1 Table

Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) belonging to several serotypes including O26:H11/H$^-$ (non-motile), O91:H21, O103:H2/H$^-$, O111:H8/H10/H$^-$, O113:H21, O145:H25/H28/H$^-$, and sorbitol-fermenting O157:H$^-$ cause watery and bloody diarrhoea (haemorrhagic colitis) and haemolytic uraemic syndrome (HUS), the trias of microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure. In Germany, non-O157:H7 EHEC serotypes are the causative organism in approximately 50 % of cases of HUS. Molecular analysis demonstrates that non-O157:H7 EHEC possess a variety of potential virulence genes in addition to Shiga toxin. These elements are believed to play a major role in the evolution and in plasticity of the EHEC genomes and contribute, via a horizontal gene transfer, to the emergence of new EHEC clones highly pathogenic for humans.

Zusammenfassung

Enterohämorrhagische *Escherichia coli* (EHEC) der Serovare O26:H11/H$^-$ (unbeweglich), O91:H21, O103:H2/H$^-$, O111:H8/H10/H$^-$, O113:H21, O145:H25/H28/H$^-$ sowie Sorbitol-fermentierende O157:H$^-$ verursachen wässrige oder blutige Durchfälle (hämorrhagische Kolitis). Als schwere Komplikation kann ein hämolytisch-urämisches Syndrom (HUS) auftreten, verbunden mit hämolytischer Anämie, Thrombozytopenie und Nierenversagen. In Deutschland sind non-O157:H7 EHEC an ca. 50 % der Fälle von enteropathischem HUS beteiligt. Molekulare Analysen zeigen, dass diese Serovare außer den Shiga-Toxinen noch weitere potentielle Virulenzfaktoren besitzen. Vergleichende Genomanalysen erlauben Rückschlüsse auf die Evolution dieser Krankheitserreger und zeigen, wie durch horizontalen Gentransfer neue und besonders virulente Varianten entstehen können.

1. Non O157:H7 EHEC in Human Disease

Besides enterohaemorrhagic *Escherichia coli* (EHEC) of serotype O157:H7 which is the most frequent EHEC associated with human disease worldwide (TARR et al. 2005), several non-O157:H7 EHEC serotypes have recently emerged as human pathogens. These serotypes, which include O26:H11/H$^-$ (non-motile), O91:H21, O103:H2/H$^-$, O111:H8/H$^-$, O113:H21, O145:H25/H28/H$^-$, and sorbitol-fermenting (SF) O157:H$^-$, have become an important cause of diarrhoea, bloody diarrhoea and haemolytic uremic syndrome (HUS) (ELLIOT et al. 2001, SONNTAG et al. 2004, BIELASZEWSKA et al. 2005c, BROOKS et al. 2005, KARCH et al. 2005, MELLMANN et al. 2005, ZHANG et al. 2007). HUS is a leading cause of

acute renal failure in children. It results from the microvascular endothelial injury in the kidneys mediated by Shiga toxins, which are the major virulence factors of EHEC (Tarr et al. 2005). In Germany, approximately 50 % of paediatric HUS cases have been associated with non-O157:H7 EHEC serotypes (Bielaszewska et al. 2007). Because of the increasing medical importance of non-O157:H7 EHEC, several investigations dealt during the last decade with the molecular and evolutionary mechanisms contributing to the emergence of these pathogens and their high virulence for humans.

2. Methods in Molecular Biology for Investigating Diversity in Non-O157:H7 EHEC

Although two complete *E. coli* O157:H7 genome sequences are currently available, knowledge on the genome content and the diversity of non-O157 EHEC has only recently started to accumulate. With the development of new methods in molecular biology, e. g. DNA sequencing, restriction fragment length polymorphism (RFLP) analysis, suppression subtractive hybridization and comparative DNA hybridization, data on the gene content became available. Moreover, by using microarrays, containing the genome of *E. coli* K-12, and a "pathoarray", which contains most of the currently known virulence genes (Dobrindt et al. 2003), different non-O157:H7 EHEC could be compared. Parallel studies on the phylogenetic grouping using multilocus sequence typing (MLST) (Wirth et al. 2006) and model cell cultures with intestinal epithelial cells and endothelial cells were used to assess host and tissue specificity and the mode of action of selected virulence factors (Janka et al. 2002,

Tab. 1 Virulence and fitness genes of the most common non-O157:H7 EHEC serotypes

Gene or gene cluster	Predicted product or phenotype[1]	Presence of the gene in EHEC of serotype				
		O26:H11/H$^-$	O103:H2/H$^-$	O111:H8/H$^-$	O145:H28/H$^-$	SF O157:H$^-$
fliC	Flagellin subunit	H11	H2	H8	H28	H7
eae (type)	Intimin	+ (β)	+ (ε)	+ (γ2)	+ (γ)	+ (γ)
EHEC-*hlyA*	EHEC hemolysin	+	+	+	+	+
cdt-V	CDT-V	–	–	–	–	+
iha	Iha	+	–	+	+	–
sfpA	Sfp fimbriae	–	–	–	–	+
efa1	Efa1	+	+	+	+[2]	+
sen	ShET2 homologue	+	+	+	+	+
pagC	PagC homologue	–	–	+	+[3]	+
ter	Tellurite resistance	+	+[3]	+	+	–
ure	Urease	+	+[3]	+	+	–
irp2/fyuA^g	Iron uptake	+	–	–	–	–

[1] CDT-V, cytolethal distending toxin V; Iha, iron-regulated gene A homologue adhesin; Efa1, EHEC factor for adherence; ShET2, *Shigella flexneri* enterotoxin 2; PagC, protein encoded by the *phoP*-activated gene C of *Salmonella enterica* serovar *Typhimurium*.
[2] Strains O145:H28/NM contain either complete *efa1* or only the 5´ *efa1* region. All the other serotypes contain a complete *efa1* gene.
[3] Only a minority of isolates contain the gene.

2003, Bielaszewska et al. 2005a, Sonntag et al. 2005, Aldick et al. 2007). The data from these experiments have added to our knowledge on the prevalence and occurrence of factors determining virulence in non-O157 EHEC. The major non O157:H7 EHEC serotypes and a list of their potential virulence and fitness factors are shown in Table 1.

3. Variations in the Flagellin Encoding Gene (*fliC*) in Non-O157:H7 EHEC

Cultures of most of the important non-O157:H7 EHEC show growth of non-flagellated or poorly flagellated organisms thus making it impossible to carry out classical serotyping (Sonntag et al. 2004, Bielaszewska et al. 2005c, Zhang et al. 2007). The inability to assess H antigen using serotyping makes it difficult to study the epidemiology of the infections, to identify reservoirs and sources of infection for humans and to determine the modes of transmission. Analysis of 72 clinical isolates of EHEC O111 from patients with diarrhoea and HUS in Germany showed that only six were motile (Zhang et al. 2007). The remaining 66 strains (92 %) were non-motile (O111:H$^-$), and thus could not be typed using conventional H serotyping (Zhang et al. 2007). Similarly, the analysis of 120 EHEC O145 isolated from patients revealed that three isolates belonged to serotype O145:H28, one to serotype O145:H25 and 116 (97 %) were non-motile (Sonntag et al. 2004). Also, a high proportion (approximately 50 %) of EHEC O26 (Bielaszewska et al. 2005c) and all SF EHEC O157 are non-motile (Mellmann et al. 2005, Bielaszewska et al. 2007). Since the flagellum apparatus is complex and consist of about 40 genes, there are many possibilities for non-expression including frame shift mutations in regulatory genes and integration of IS elements. The variability in the H antigen is associated with variability in the amino acid sequence of the flagellin and with the variability in nucleotide sequence in its structural gene (*fliC*). The 5´ and 3´ portions of *fliC* are highly conserved whereas the middle section shows significant variability and encodes portions of the H type-specific surface proteins. Targeting the highly variable region by PCR provides the basis for distinguishing between the various *fliC* genes using RFLP analysis of the PCR product (Machado et al. 2000, Prager et al. 2003, Sonntag et al. 2004, Zhang et al. 2007). For example, in our recent study, EHEC O111:H$^-$ strains displayed three different *fliC*-RFLP patterns that were identical to those of motile EHEC O111 with H antigens H 8, H10, and H11 (Zhang et al. 2007). The H8 and H11 *fliC*-RFLP patterns were associated with *eae* γ2 and *eae* β, respectively, whereas the H10 *fliC* genotype was present only in *eae*-negative EHEC O111 strains (Zhang et al. 2007). In EHEC O145, two *fliC*-RFLP patterns were observed (Sonntag et al. 2004). The majority of strains displayed the H28 *fliC*-RFLP pattern and harbored *eae* γ, whereas a small proportion of strains with the H25 *fliC*-RFLP pattern possessed *eae* β (Sonntag et al. 2004). Among EHEC O103, the most common H type and *fliC*-RFLP type is H2 which is associated with *eae* ε (Beutin et al. 2004, Bielaszewska et al. 2007) but strains with H/*fliC*-RFLP types H11, H18, and H25 have also been isolated from patients (Schimmer et al. 2008). These data show the existence of at least two (EHEC O145), three (EHEC O111) or four (EHEC O103) clonal lineages. In contrast, all motile (H11) and non-motile EHEC O26 strains analyzed up to now possess $fliC_{H11}$ and *eae* β (Bielaszewska et al. 2005c) indicating that they all belong to a common clone complex. The clonal diversity within certain non-O157 EHEC serogroups assists us in the molecular typing of these emerging pathogens and contributes to our understanding of their clinical importance and epidemiology.

4. Comparative Genomics of Non-O157:H7 EHEC

The pathoarray first described by Dobrindt et al. (2003) is a reliable tool for the rapid and comprehensive identification of virulence genes in an *E. coli* strain and thus for assessing the degree of infectivity. After sequencing a cosmid library from SF EHEC O157:H$^-$ strain 493/89 and a large plasmid from SF EHEC O157:H$^-$ strain 3072/96 the sequences were compared with publicly available *E. coli* genome sequences and screened for candidate determinants according to the known and anticipated functions. With the aid of these methods we were able to show that a variety of disease- and fitness-associated loci, present in *E. coli* O157:H7, are missing in SF *E. coli* O157:H$^-$ and several loci present in SF *E. coli* O157:H$^-$ are missing from *E. coli* O157:H7 (Janka et al. 2002, 2003, Bielaszewska et al. 2005b, Friedrich et al. 2004, 2005, Janka et al. 2005, Brunder et al. 2006, Orth et al. 2006, 2007). The composition of a mosaic island present in SF EHEC O157:H$^-$ which is composed of fragments of the genome of EHEC O157:H7 strain EDL933 and the *Shigella* resistance locus (SRL) is shown in Figure 1. Typical virulence and fitness genes of SF EHEC O157:H$^-$ are listed in Table 1. It can be concluded that the accumulation of virulence-associated sequences determines the pathotype and that marked heterogeneity is present in the gene content of *E. coli* strains and even in the gene content of members of the same serotype.

Non-O157 EHECs are thought to contain genes conferring fitness, toxicity and colonization properties giving these organisms advantages over commensals. These subsidiary genes are organized as a mosaic of DNA islands within the conserved backbone. Genomic

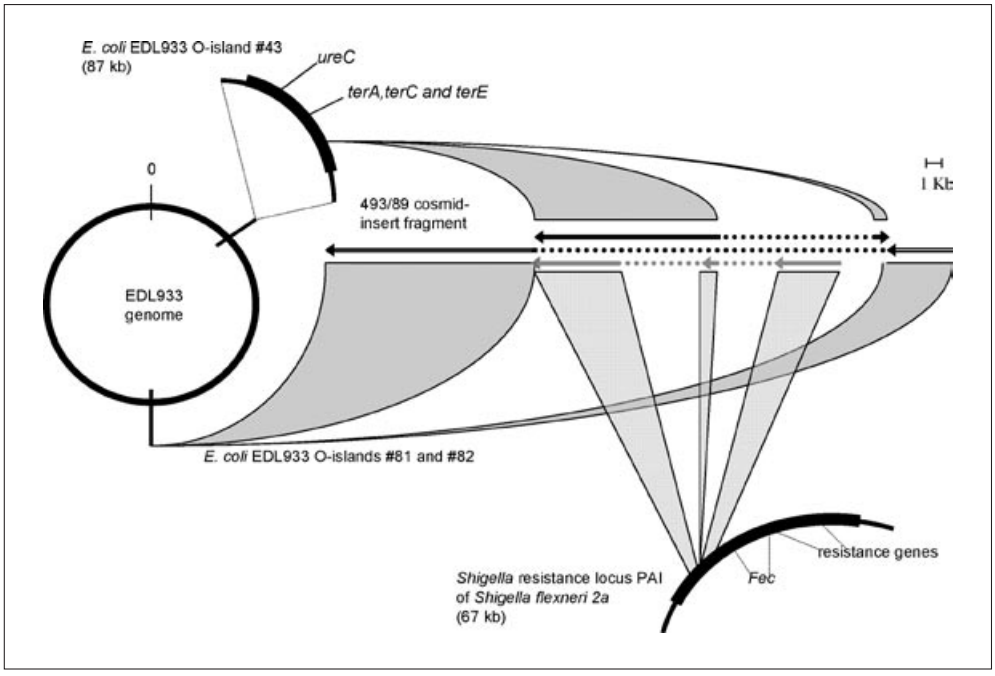

Fig. 1 Structure of a mosaic island of SF EHEC O157:H$^-$ which is composed of fragments of the genome of *E. coli* O157:H7 EDL933 and the *Shigella* resistance locus (SRL)

islands containing the virulence genes are known as pathogenicity islands (Dobrindt et al. 2004), and these have the potential to undergo rearrangements, deletions and insertions (Dobrindt et al. 2004, Ahmed et al. 2008). Studies on the genomic composition of EHEC O157:H7 and SF EHEC O157:H$^-$ have identified differences in potential virulence genes. For example, in SF EHEC O157:H$^-$ strains there is a large gene, *efa1* (Janka et al. 2002) originally identified in an EHEC O111:H$^-$ strain (Nicholls et al. 2000) which is essential for adherence of the organism to cultured epithelial cells, for haemagglutination and autoaggregation. The product of the *efa1* gene, Efa1, is highly homologuos with LifA, a toxin produced by EPEC that specifically inhibits lymphocyte proliferation and production of interleukin-2, interleukin-4 and γ-interferon in mitogen-stimulated lymphocytes (Klapproth et al. 2000). Efa1 and LifA belong to a family of large toxins which includes clostridial cytotoxins and possess a 500 amino acid region highly homologous with toxin B of *Clostridium difficile*. We have shown (Janka et al. 2002) that SF EHEC O157:H$^-$ possess the complete *efa1* open reading frame that is present in only a rudimentary form in EHEC O157:H7 (Perna et al. 2001). The presence of the complete *efa1/lifA* gene can be demonstrated using PCR analysis with primers complementary to three different regions of the gene (Janka et al. 2002). All SF EHEC O157:H$^-$ strains examined so far in our laboratory using this method possess the complete gene but this gene was not found in EHEC O157:H7 strains (Bielaszewska et al. 2007). A complete *efa1* gene is also present in EHEC O26:H11/H$^-$ (Bielaszewska et al. 2005c, Bielaszewska et al. 2007), O103:H2 (Bielaszewska et al. 2007), O145:H28 (Sonntag et al. 2004) and O111:H8/H11 but not in EHEC O111:H10 (Zhang et al. 2007).

The role of Efa1 in the pathogenesis of infections caused by EHEC is not clear. Efa-1 has been shown to enhance colonization of the bovine intestine by EHEC O111 (Stevens et al. 2002), suggesting that it can act as an adhesin. In a recent report Efa1 from EHEC O103:H2 inhibited mitogen-activated proliferation of bovine peripheral blood lymphocytes, indicating its role as lymphostatin (Abu-Median et al. 2006). Non-O157:H7 EHEC harboring *efa1* investigated in our laboratory do not display autoaggregation, a characteristic which has been linked with the presence of *efa1* in EHEC O111 (Nicholls et al. 2000). However, these organisms adhere well to human intestinal epithelial cell lines HCT-8 and T84 cells (Aldick et al. 2007). Further investigations using mutants lacking functional Efa1 are necessary to determine the role of this protein during EHEC infection.

In addition to *efa1*, which is located in a homologue of O island 122 (Perna et al. 2001) in EHEC O26, 111 and 145, marker genes for O islands 43 and 48 in of EHEC O157:H7 strain EDL933 (Perna et al. 2001) have been identified in EHEC of the major non-O157 serogroups such as O26, O103, O111, and O145. These include the *ter* gene cluster conferring tellurite resistance (Bielaszewska et al. 2005b, Zhang et al. 2007, Orth et al. 2007) and the *ure* gene cluster encoding urease (Friedrich et al. 2006, Orth et al. 2006, Zhang et al. 2007). However, these genes are absent from SF EHEC O157:H$^-$ (Bielaszewska et al. 2005b, Friedrich et al. 2005, Orth et al. 2006, 2007). Another genomic island termed high pathogenicity island (HPI), which encodes an iron uptake system is usually present in EHEC O26:H11/H$^-$ where it could function as a fitness island (Karch et al. 1999), but is absent from EHEC O103, O111 and O145 (Karch et al. 1999).

Taken together, these data demonstrate that non-O157:H7 EHEC possess a variety of potential virulence and fitness genes in addition to Shiga toxin. The spectra of these genes and the microbe-host interactions appear to be serotype-specific or strain-specific. The iden-

tification of the non-Shiga toxin virulence and fitness genes and analysis of the mobile genetic elements (pathogenicity and fitness islands, bacteriophages, plasmids) which encode these loci and which have been shown to play a major role in the evolution and plasticity of the *E. coli* genome (Dobrindt et al. 2004, Ahmed et al. 2008) have provided a major insight into the emergence of the non-O157:H7 EHEC human pathogens.

5. Future Perspectives

The considerable public health problem caused by non-O157:H7 EHEC and the lack of a specific therapy for these infections necessitates

(*i*) the development of effective preventive measures,
(*ii*) an improvement in our knowledge of the epidemiology and evolutionary mechanisms underlying the emergence of these pathogens, and
(*iii*) the introduction of new methods for their molecular typing.

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Special Conditions Allow Binding of the Siderophore Salmochelin to Siderocalin (NGAL-Lipocalin)

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With 2 Figures

Abstract

Siderocalin is part of the innate immune system and is secreted by epithelial cells in the early stages of inflammation. This protein binds catecholate siderophores such as enterobactin with high affinity. As a consequence, the iron supply and growth of the siderophore-producing bacteria is disturbed. Recombinant siderocalin isolated from strains of *Escherichia coli* K-12 contained bound enterobactin. Very low amounts of siderocalin were isolated from another K-12 strain, unable to produce enterobactin, which indicated that enterobactin might stabilize the recombinant protein. Siderocalin isolated from *E. coli* strain Nissle 1917, which produces the glucosylated enterobactin salmochelin, contained a mixture of bound salmochelin (55 %) and enterobactin (45 %). Growth of an enterobactin-producing *E. coli* K-12 strain, but not of the same strain carrying a plasmid encoding *iroBCDEN* and therefore able to produce salmochelin, was suppressed when siderocalin was added to the medium, which indicated that salmochelin is bound by siderocalin before it folds into its final conformation in the cell and that binding of salmochelin to matured siderocalin is not possible. Salmochelin is mainly produced by pathogenic enterobacteria. Glucosylation of enterobactin may be a mechanism by which these pathogenic bacteria evade trapping of enterobactin by siderocalin and get their iron for growth.

Zusammenfassung

Siderocalin ist ein Bestandteil des angeborenen Immunsystems und wird in den frühen Stadien der Entzündung durch Epithelzellen sezerniert. Dieses Protein bindet Katecholat-Siderophore wie Enterobaktin mit hoher Affinität. Daraus resultiert eine Störung der Eisenversorgung und des Wachstums von Siderophor-produzierenden Bakterien. Rekombinantes Siderocalin, das von *Escherichia coli* K-12-Stämmen isoliert wurde, enthielt gebundenes Enterobaktin. Von einem K-12 Stamm, der kein Enterobaktin produzieren kann, wurden nur geringe Mengen Siderocalin isoliert, was für eine Stabilisierung des rekombinanten Proteins durch Enterobaktin sprechen würde. Der *E. coli*-Stamm Nissle 1917 produziert das glykosylierte Enterobaktin Salmochelin. Von diesem Stamm isoliertes Siderocalin enthielt einen Mix aus gebundenem Salmochelin (55 %) und Enterobaktin (45 %). Das Wachstum eines Enterobaktin-produzierenden *E. coli* K-12-Stammes wurde durch die Zugabe von Sidercalin ins Medium gehemmt, nicht jedoch das Wachstum des gleichen Stammes, der ein Plasmid enthält, das *iroBCDEN* kodiert und daher Salmochelin produzieren kann. Dies deutet darauf hin, dass Salmochelin durch Siderocalin gebunden wird, bevor dies seine endgültige Konformation in der Zelle annimmt, und dass die Bindung von Salmochelin an das reife Sidercalin nicht möglich ist. Die Glykosylierung des Enterobaktins könnte ein Mechanismus der pathogenen Bakterien sein, um dem Abfangen des Enterobaktins durch Siderocalin zu entgehen und so die Eisenversorgung zu sichern.

1. Introduction

Iron is an essential element for bacterial growth. In mammalian blood you find only a minimum of free iron accessible for bacteria. Therefore pathogenic bacteria often produce specific iron-binding agents, known as siderophores that are excreted and bind free and bound iron Fe^{3+} with affinities in the nanomolar range (Braun et al. 1998). Enterobactin is the chelator produced by *Escherichia coli*. This bacterial iron supply system is interrupted by the high affinity of the host's siderocalin for iron-enterobactin. Siderocalin is important for defense against enterobactin-producing bacteria; after i. p. challenge with an enterobactin-producing *E. coli* strain, 80 % of siderocalin-deficient mice died within 42 h, whereas all siderocalin-proficient mice survived (Flo et al. 2004). *Salmonella enterica* had long been assumed to secrete enterobactin as a major siderophore under low-iron conditions. It has been recently shown that *S. enterica* and certain uropathogenic *E. coli* strains produce a twofold C-glucosylated enterobactin, called salmochelin (Bister et al. 2004, Hantke et al. 2003) which is discussed to be seen as an evasion mechanism of these bacterial strains.

2. Results

Salmochelin S4 is bound by siderocalin if siderocalin is heterologously expressed in salmochelin producing *E. coli*, and enterobactin might stabilize recombinant produced siderocalin (Fig. 1).

Enterobactin producing strains were inhibited in their growth by siderocalin in an iron poor medium, whereas salmochelin producers were not. *In vitro* salmochelin S4 is not able to replace enterobactin bound to siderocalin (Fig. 2).

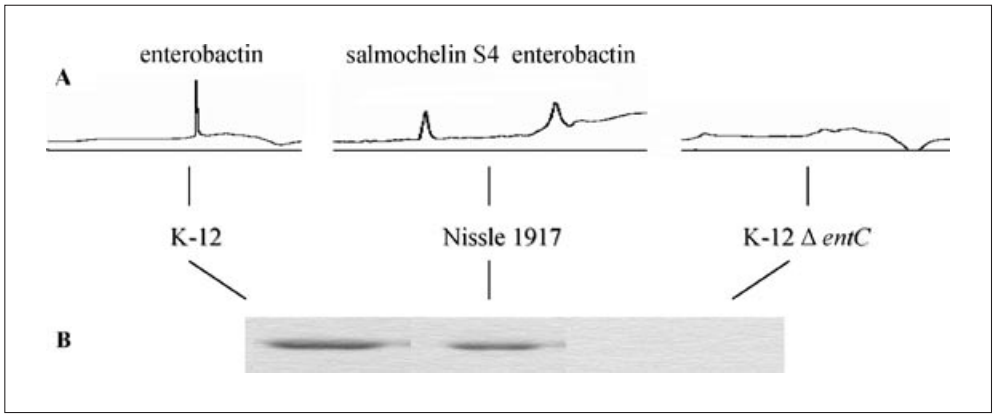

Fig. 1 Siderophore binding to GST-siderocalin. (*A*) GST-siderocalin was isolated from *E. coli* K-12 (pGEX-4T3-NGAL) which contained bound enterobactin. GST-siderocalin isolated from Nissle 1917 (pGEX-4T3-NGAL, pGP1–2) contained bound enterobactin and salmochelin (45 % to 55 %). Only very low amounts of siderocalin were isolated from *E. coli* K-12 Δ *entC* (pGEX-4T3-NGAL, pGP1–2). The siderophore content was analyzed by HPLC. Since very low amounts of siderocalin were isolated from *E. coli* K-12 Δ *entC* and the mutant is unable to produce enterobactin, no siderophores could be detected. (*B*) SDS-PAGE of purified GST-siderocalin from strains indicated.

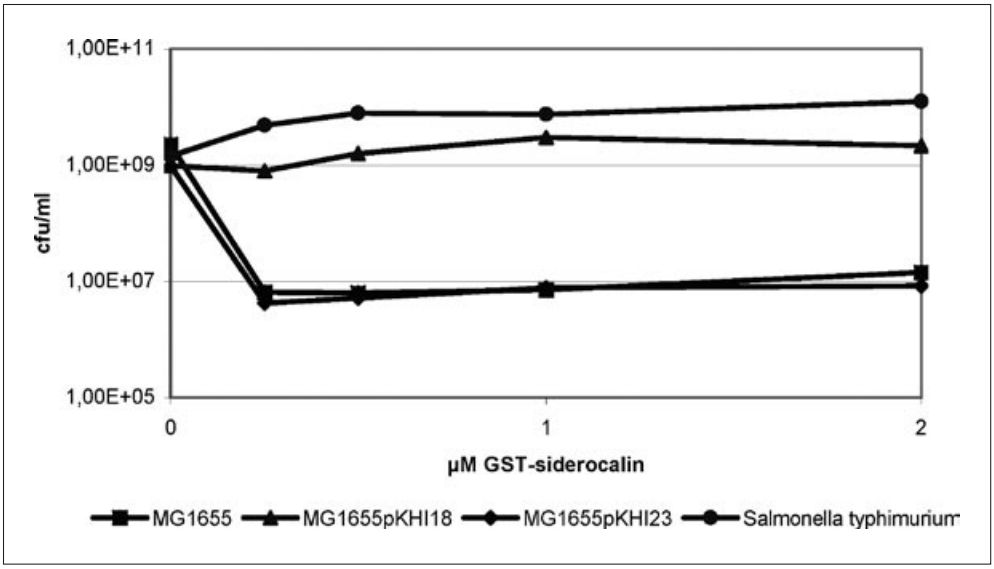

Fig. 2 Growth in the presence of GST-siderocalin. Recombinant siderocalin was isolated as a glutathione-S-transferase (GST) fusion protein from *Escherichia coli* K-12 strains. GST-siderocalin contained bound enterobactin, however, it was in most cases only partially saturated with enterobactin (about 30 %). Addition of this GST-siderocalin to *E. coli* K-12 in an iron poor medium inhibited growth of this strain (squares). When *E. coli* K-12 contained the plasmid pKHI18, encoding the *iroA* locus (*iroBCDEN)* which allows synthesis of salmochelin and utilization of Fe-salmochelin, the strain was able to grow in the presence of GST-siderocalin (triangle). *Salmonella enterica* producing salmochelin was also able to grow in the presence of GST-siderocalin (filled circle). *E. coli* K-12 containing the plasmid pKHI23, encoding the receptor IroN and the two esterases IroE and IroD necessary for the uptake and utilization of Fe-salmochelin, is not able to produce salmochelin. This strain (diamonds) was not able to grow in presence of GST-siderocalin, just like K-12 without any plasmid.

3. Conclusions

Siderocalin is secreted during inflammation into serum (Flo et al. 2004) and also into urine as an early marker of kidney injury (Trachtman et al. 2006). One function of siderocalin in the urine might be to suppress catecholate-producing bacteria. Since growth of salmochelin-producing bacteria is not inhibited by siderocalin (Valdebenito et al. 2007) glucosylation of enterobactin to form salmochelin might be an evasion mechanism of certain pathogenic bacteria, e. g. *Salmonella enterica*, urinary-tract-infecting *E. coli*, and other urinary-tract-infecting *Enterobacteriaceae*. Similar observations were published by (Fischbach et al. 2006).

Urine is an iron-poor medium, and the majority of *E. coli* strains growing in this environment produce 4 different siderophores: enterobactin, salmochelin, aerobactin, and yersiniabactin (Valdebenito et al. 2006). In addition to 11 defined siderophore/heme receptors, these urinary-tract-infecting *E. coli* strains encode up to 6 putative siderophore/heme receptors in their outer membrane, which may help to provide iron. *E. coli* K-12 and many other, often pathogenic *E. coli* strains have only 7–10 more-or-less well-characterized siderophore/heme receptors. This is an indication that the iron supply in urine is an important

factor for bacterial growth. Work is in progress to investigate whether one of these TonB-dependent putative siderophore receptors is a siderocalin receptor that could allow the bacterium to gain back iron-enterobactin from siderocalin.

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Identification of an Avian Pathogenic *Escherichia coli* Metabolic Region Implicated in Host Adaptation

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With 1 Figure and 1 Table

Abstract

By substractive hybridization between the genomes of an Avian Pathogenic *E. coli* (APEC) strain and of a non-pathogenic *E. coli* strain of avian origin, we identified a 7400 bp region (*frz*) that is absent from the non-pathogenic strain. This region is very rarely present in non-pathogenic strains of avian origin. The prevalence of *frz* in APEC strains increases with their virulence. *Frz* was sequenced in an APEC strain (BEN2908) of the most virulent group and found to contain eight genes coding for the three subunits of a PTS transporter from the fructose sub-family, for a transcriptional activator of PTS systems, for two type II ketose-1,6-biphosphate aldolases, for a sugar specific kinase (ROK family) and for a protein of unknown function. In strain BEN2908, these eighth genes are expressed as a polytranscript from a promoter localized upstream of the first open-reading frame (coding for the PRD-regulator). All these genes are transcribed during the complete *in vitro* growth-phase. We have deleted the entire *frz* region of strain BEN2908 and inserted a kanamycin resistance cassette at that position. We fed axenic White Leghorn chickens of twelve days with a mixed bacterial culture containing strain BEN2908 and its isogenic deletion mutant. The evolution of the proportion of these strains in the intestinal tract of the chickens was followed during seven days. This experiment showed that the *frz* region procures an advantage to strain BEN2908 for the colonization of the chicken intestinal tract. Mixed cultures of strain BEN2908 and of its isogenic deletion mutant in LB medium and in condition of partial anaerobiosis (condition close to that found in the intestinal tract) also indicate that this region gives an advantage to BEN2908 during the stationary *in vitro* growth-phase. Our data, connecting the level of virulence of the strains with their metabolic capacities, open interesting perspectives on the comprehension of bacterial virulence mechanisms.

Zusammenfassung

Mit Hilfe der subtraktiven Hybridisierung der Genome eines vogelpathogenen *E. coli* (APEC)-Stammes und eines apathogenen *E. coli*-Vogelisolates konnten wir eine 7400 bp große Region (*frz*) identifizieren, die in dem apathogenen Stamm nicht vorkommt. Diese Region kommt in apathogenen Vogelstämmen sehr selten vor. Die Verbreitung der *frz*-Region in APEC-Stämmen steigt mit zunehmender Virulenz. Die *frz*-Region des APEC-Stammes BEN2908, der zur virulentesten Gruppe der APECs zählt, wurde sequenziert. Sie enthält acht Gene, die für die drei Untereinheiten des PTS-Transporters der Fructose-Unterfamilie kodieren sowie für einen transkriptionellen Aktivator des PTS-Systems, für zwei Typ-II-Ketose-1,6-Biphosphat-Aldolasen, für eine Zucker-spezifische Kinase (der ROK-Familie) und für ein Protein unbekannter Funktion. Diese acht Gene werden im Stamm BEN2908 als Polytranskript von einem Promotor aus transkribiert, der stromaufwärts vom ersten offenen Leserahmen (dieser kodiert den PRD-Regulator) lokalisiert ist. All diese Gene werden während der gesamten *In-vitro*-Wachstumsphase transkribiert. Wir haben die gesamte *frz*-Region im Stamm BEN2908 deletiert und stattdessen eine Kanamyzin-Kassette inseriert. Keimfreie weiße Leghorn-Hühner von 12 Tagen wurden mit einer gemischten Bakterienkultur gefüttert, die den Stamm BEN2908 und seine isogene Deletionsmutante enthielt. Die Entwicklung des Mengenverhältnisses der beiden Stämme im Verdauungstrakt der Hühner wurde über einen Zeitraum von sieben Tagen beobachtet. Dieses Experiment zeigte, dass die *frz*-Region für den Stamm

BEN2908 für die Besiedelung des Verdauungstraktes der Hühner von Vorteil ist. Mischkulturen des Stammes BEN2908 und seiner isogenen Deletionsmutante, die in LB-Medium und unter teilweiser Anaerobiose (Bedingungen, die denen im Verdauungstrakt sehr ähnlich sind) gehalten wurden, zeigen ebenfalls, dass diese Region für BEN2908 während der stationären Wachstumsphase unter *In-vitro*-Bedingungen einen Vorteil bietet. Unsere Daten eröffnen interessante Perspektiven für unser Verständnis von bakteriellen Virulenzmechanismen, da sie eine Verbindung zwischen dem Virulenzgrad der Stämme und ihren Stoffwechseleigenschaften herstellt.

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) are responsible for a variety of human and animal diseases. Their infection process is multifactorial and still incompletely known. ExPEC strains are divided in several pathotypes responsible for urinary tract and meninges infections of human (UPEC and MENEC pathotypes, respectively), and extra-intestinal infections of all avian species (APEC pathotype). ExPEC are also responsible for septicemia in human and animal. Actually, the MENEC pathotype is the second cause of neonatal meningitis (25–30 % of cases), after the group B *Streptococcus* (May et al. 2005). Urinary tract infections caused by UPEC are the most common infections due to bacteria (40 %), they are responsible for 3.5 millions consultations of physician each year in Europe (Hooton and Stamm 1997, Russo and Johnson 2006). Today, the APEC pathotype is one of the main causes of economical losses due to bacteria in the poultry industry and of condemnation at slaughter-house (Dho-Moulin and Fairbrother 1999). APEC strains are phylogenetically linked with strains from other pathotypes of the ExPEC group with which they share numerous virulence factors. This suggests that the APEC strains could represent a zoonotic risk (Moulin-Schouleur et al. 2007).

APEC strains are inhabitants of the intestine of many avian species. They are also responsible for a systemic disease in chickens and turkey chicks that starts with a respiratory tract infection. This infection is usually secondary to immunosuppression or damaged of the respiratory tract by viral (infectious bronchitis virus, hemorrhagic enteritis virus or Newcastle disease virus) or mycoplasmal infections, or by environmental stresses (exposure to ammonia and dust). APEC infections in poultry occur by inhalation of feces-contaminated dust. APEC first reach the upper respiratory tract, then colonize the lower respiratory tract and gain access to the air sacs and lungs. They finally gain entry into the blood stream and induce a systemic infection by colonizing internal organs such as the hearth, the liver and the spleen. The most frequent syndromes of APEC infection in poultry are aerosacculitis, pericarditis, perihepatitis and septicemia (Dho-Moulin and Fairbrother 1999).

Various virulence factors of APEC were described. Those factors are nevertheless present in variable combinations among these strains. Fimbrial or afimbrial adhesins are implicated in the colonization of poultry. Type I fimbriae have a major role in APEC adherence to the chicken intestine, whereas type P fimbriae (*pap* operon) are expressed during colonization of the air-sacs, the lung and the internal organs. Iron-sequestering systems (aerobactin, Iro proteins, yersiniabactin, Sit system) allow the multiplication of the bacteria in low iron concentration environments. Other factors, such as anti-host defense factors (OmpA, Iss, LPS, K1 capsule), autotransporters (Tsh and VAT), IbeA protein, and proteins implicated in carbohydrate metabolism (MFS transporter, glycosidase, LacI transcriptional regulator) were also described. Some of those factors are encoded by pathogenicity islands (PAI) inserted at tRNA loci. This is the case for the VAT protein coded by a PAI inserted at the *thrW* tRNA locus of strain EC222, for the *pap* operon identified in a PAI inserted at the *pheV* locus of strain APEC-01, and for proteins implicated in carbohydrate metabolism coded by

the AGI-3 PAI inserted at the *selC* tRNA locus of strain BEN2908. Nevertheless, the above components cannot explain all the disease process which is complex and multi-factorial (Chouikha et al. 2006, Dho-Moulin and Fairbrother 1999, Germon et al. 2005).

To better characterize the pathogenic mechanism of APEC strains and identify new virulence factors, a substractive hybridization between the genomes of an APEC strain and of a non-pathogenic *E. coli* strain of avian origin was made in our laboratory (Schouler et al. 2004). Among others, this experiment allowed the identification of a metabolic region that is absent from the non-pathogenic strain. We showed that this region is very rarely found among non-pathogenic strains of avian origin (less than 2 %) and that its association with APEC strains increases with their virulence, reaching an association of 74 % with the APEC strains from the most virulent group (Moulin-Schouleur and Gilot, unpublished results). The above cited metabolic region from an APEC strain of the most virulent group (BEN2908) was sequenced and found to contain eight genes potentially implicated in carbohydrate metabolism. Three of them encode a PTS transporter of the fructose sub-family (EIIA, EIIB, EIIC). The other genes code for a transcriptional activator of PTS systems (protein with PRD domains), two type II ketose-1,6-biphosphate aldolases, a sugar specific kinase of the ROK family and a protein of unknown function (Tab. 1). None of these proteins is actually described in the literature. To be conformed to the nomenclature of PTS transporters of the fructose sub-family, we named this new transporter, Frz, and the all metabolic region, *frz* region. Blast homology searches indicated that the entire *frz* region is absent from the genome of the two sequenced *E. coli* K-12 non-pathogenic strains. In the APEC strain BEN2908, the eight *frz* genes are expressed in the form of a polytranscript from a promoter localized upstream of the first open-reading frame (coding for the PRD-regulator). All these genes are transcribed during the complete *in vitro* growth-phase (Rouquet and Gilot, unpublished results).

Tab. 1 Genes present in the *frz* metabolic region

ORF	Function
1	Transcriptional activator (PRD domains proteins)
2	Subunit IIA of a fructose-like PTS transporter
3	Subunit IIB of a fructose-like PTS transporter
4	Subunit IIC of a fructose-like PTS transporter
5	Class II ketose 1,6-biphosphate aldolase
6	Class II ketose 1,6-biphosphate aldolase
7	ROK sugar specific kinase
8	Unknown

PTS transporters and their specific regulators (regulators with PRD domains of the antiterminator or activator type) are absent from eukaryotic organism and are only present in some bacterial species. Their main function is the translocation of carbohydrates inside bacteria but more and more data from the literature indicate that these systems are also probably, as two-component systems, environmental sensors affecting diverse physiological aspects of the bacteria, and particularly the expression of their virulence. The PTS system is composed of enzyme I (EI), Hpr (heat stable histidin protein), and enzyme II (EII). EI and Hpr

are general cytoplasmic energy-coupling proteins which lack sugar specificity. EII is made of several domains (EIIA, EIIB, EIIC and sometimes EIID) and has the function of a carbohydrate permease specific for one or a few sugar. EIIA and EIIB are cytoplasmic domains, whereas (EIIC and EIID) are membrane domains. All these domains can eventually be fused in one or several proteins. Phosphoryl relay, energizing the transporter, proceeds sequentially from PEP to EI, Hpr, EIIA, EIIB, EIIC, and, finally, the incoming sugar which is transported across the internal membrane via the integral membrane EIIC porter. The level of phosphorylation of PTS components influences the level of phosphorylation of PRD regulators. Following the phosphorylation level of these regulators, operons coding PTS transporters and some virulence genes are activated or not (Deutscher et al. 2006, Stulke et al. 1998, Tsvetanova et al. 2007).

The high association of the *frz* region to APEC strains and our *in silico* studies suggest non-exclusively that:

(*i*) this region gives a metabolic advantage to the strains for the colonization of the intestinal tract and/or target organs of extra-intestinal colibacillosis;
(*ii*) the PTS transporter encoded by the region is a sensor of the environment and it regulates virulence and/or host adaptation genes by phosphorylation/dephosphorylation mechanisms implicating its EIIA and EIIB subunits and the PRD-regulator.

As a first approach to these questions, we have deleted the entire *frz* region of the APEC strain BEN2908 and inserted a kanamycin resistance cassette at this position (Barra and

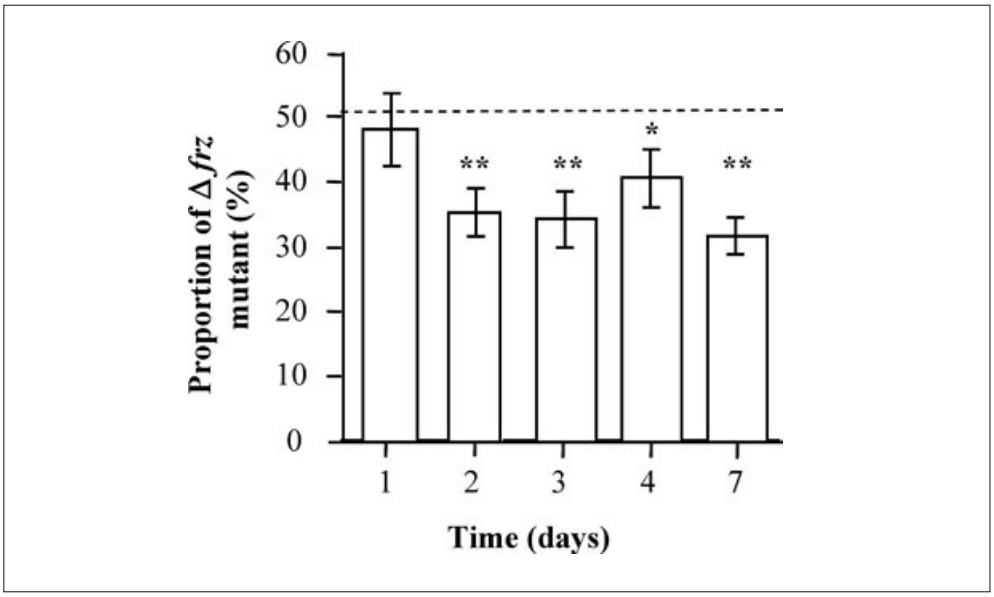

Fig. 1 Colonization of the intestinal tract of chicks with a mixed culture of strain BEN2908 and BEN2908Δ*frz*::kan. Six axenic White Leghorn chicks of 12 days were fed with a mixed culture of strains BEN2908 and BEN2908Δ*frz*::kan. The proportion of the mutant in faeces of the chicks was analyzed during 7 days. The proportion of the mutant in the inoculum is 51 % (- -). Data are presented as the means ± standard deviations for the six animals analyzed. Signification of the results were tested with a t Student test (**, $P < 0.001$; *, $0.01 > P > 0.001$).

Gilot, unpublished results). We then fed axenic White Leghorn chicks of twelve days with a mixed bacterial culture containing strain BEN2908 and its isogenic mutant Δ*frz*::kan. The evolution of the proportion of these strains in the intestinal tract of these animals was followed during seven days. Data presented in Figure 1 indicate that the *frz* region gives an advantage to strain BEN2908 for the colonization of the chick intestinal tract. Mixed cultures of strain BEN2908 and of its isogenic mutant Δ*frz*::kan made in LB medium and condition of partial anaerobiosis (condition close to that found in the intestinal tract) also indicates that this region gives an advantage to BEN2908 during the stationary *in vitro* growth-phase, whereas this effect is not observed during the exponential growth-phase (Rouquet and Gilot, unpublished results).

In conclusion, we have identified a genomic region promoting the adaptation of an APEC strain to its host. The functional characterization of the diverse proteins coded by this metabolic region and the determination of their role in the pathogenic mechanism of *E. coli* is under study in our laboratory. Our data, connecting the level of virulence of the strains with their metabolic capacities, open interesting perspectives on the comprehension of bacterial virulence and host adaptation mechanisms.

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The Pathogenic Potential of *Escherichia coli* – Closing Remarks

Volkmar Braun ML (Tübingen)

The outstanding conference once more demonstrated the attractiveness of *Escherichia coli* as a model organism for studying microbial pathogenicity, including genomics and gene regulation, virulence factors, and host-pathogen interactions and emphasized its medical importance. The conference started with an historical account of the importance of *E. coli* for the development of molecular biology which forms conceptually and experimentally the framework of the current research on microbial pathogenicity. Only by this means the complex gene expression patterns during infections can be dissected into comprehensible single events which can then be reassembled into complete phenotypes. Nevertheless, we are outwitted by *E. coli*. All the wonderful modern techniques, genomics, transcriptomics, proteomics, metabolomics, NMR, X-ray analysis, and imaging techniques are not sufficient to define with certainty a pathogen as opposed to a commensal. Virulence factors have been identified, among them dominant ones such as protein toxins, but in most cases a combination of many factors define a pathogen. *E. coli* presents itself as a genetically highly versatile organism with a conserved core of genes and a flexible gene pool that provides an impressive metabolic and pathogenic flexibility to cope with many different environments. Accordingly versatile are the phenotypes, ranging from commensal strains to strains that cause urinary tract infections, intestinal infections, sepsis, and meningitis. Much work is devoted to the fascinating question how the various pathogenic strains of *E. coli* evolved. Interactions with the host is a major issue; how bacteria avoid the immune defense, how they deal with the native and acquired immune system, how they manipulate host cells for their own multiplication in acute and chronic infections. Bacteria-induced host modifications and host-induced regulatory cascades in bacteria are being uncovered. This research does not contribute only to the understanding of pathogenicity mechanisms but provides fundamental insights into the ways prokaryotic and eukaryotic cells function. Because of the strong and highly specific effects of bacteria on host cells, bacteria serve as tools in cell research. Finally, it can be expected that the knowledge gained in fundamental and clinical research will further help to prevent infections, to provide new diagnostic tools and antibiotics, to cope with the antibiotic resistance problem, and to develop vaccines to combat bacterial infections.

In 100 excellent talks and posters most of the relevant aspects of *E. coli* pathogenicity were covered. The scope of the topics was much too broad to be summarized here. The papers in this volume summarize the present state of the particular field. Theodor Escherich

would have been surprised and impressed about the great potential of his "bacterium coli commune". The limited number of attendees offered sufficient opportunities for discussions, and there was enough space and time to give proper attention to the posters.

In the years to come when most of the scientific details will have faded away and new scientific towers are built on the ground of this conference, the conferees will vividly remember the great hospitality provided by the organizers, the impressive concert of Werner GOEBEL, the tour to the world heritage city of Bamberg, the grandiose baroque building of the Banz monastery and its beautiful surrounding.

The conferees sincerely thank Jörg HACKER and his outstanding crew, his colleagues of Würzburg, and the international scientific advisers for this wonderful scientific and personal experience.

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