PhD thesis

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Examination of bacterial growth rate during host infection and its importance for antimicrobial treatment

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2. LIST OF PAPERS

The thesis is based on the following three papers, referred to by their Roman numerals:


3. ABSTRACT

*Escherichia coli* is the leading cause of bacteraemia caused by Gram negative bacteria and the overall leading cause of urinary tract infection in humans. Bacterial infections arise when bacteria are able to evade the immune system of the host and access areas where they can proliferate. Being metabolically active is a necessity for the production of virulence factors, and most antibiotics in clinical use depend on active bacterial growth to exert their effect. However, little is known about the growth dynamics taking place during infection in the host (*in vivo*). Despite several techniques to measure *in vivo* bacterial growth rate having been proposed over the past decades, no gold standard method exists. The aim of this PhD thesis was to explore the use of molecular biology methods to measure the mode of the *E. coli* chromosome replication apparatus as readout of bacterial growth rate during host infection. The thesis is based on three papers.

In **paper I**, we demonstrate the applicability of bacterial chromosome replication status as a means to measure *in situ* growth rates of *E. coli* propagating in the peritoneum and in the blood during experimental disseminated infection in mice. From a combination of two complementary methods (real-time quantitative PCR [qPCR] and fluorescence microscopy) for differential chromosomal origin (*oriC*) and terminus (*terC*) copy number quantification (*ori:ter*) in fluorescently labelled *E. coli*, we were able to measure *in vivo* bacterial growth rate both on a population average and on a single-cell level. We demonstrate in this model: (i) that the bacterial populations propagating during infection are continually heterogenic (i.e., constituted by cells growing at different growth rates), (ii) that a complete cessation of growth does not occur, and (iii) that *E. coli* does not grow independently in the bloodstream during septicaemia.

In **paper II**, we extended the approach of probing bacterial growth rate from differential genome origin and terminus copy number quantification into exploring its potential in predicting antibiotic effect as a function of pretreatment *in situ* bacterial growth rate in the same experimental infection model. Here, we demonstrate that the activities of the β-lactam antibiotic ceftriaxone and the aminoglycoside antibiotic gentamicin were highly dependent on
active bacterial growth. The fluoroquinolone ciprofloxacin, however, was less sensitive to bacterial growth rate, as the activity remained largely unchanged in rapid, compared to slow, bacterial growth rate treatment induction in vivo. Moreover, by analysis of posttreatment or time we demonstrate that ceftriaxone and gentamicin induced preferential elimination of rapidly growing cells, whereas this was not evident for ciprofloxacin.

In paper III, we included hospitalised patients with E. coli bacteriuria in a clinical study to evaluate the use of differential genome quantification as a measure of growth rate of bacteria propagating in the human urinary tract. We were able to track bacterial growth rates in patients both with and without (i.e., asymptomatic bacteriuria) symptoms of urinary tract infection (UTI) for up to four consecutive days. We observed active bacterial growth in the majority of the urine samples. There were somewhat higher bacterial growth rates in patients with UTI (compared to patients without) and in patients with invasive infection (compared to patients without), suggesting that active bacterial growth could be a factor contributing to pathogenicity. However, these observations warrant future evaluation in a larger cohort.

In summary, the findings reported in these papers provide insight into the bacterial growth dynamics taking place during host infection and demonstrate the contribution of bacterial growth rate to antibiotic effect in vivo.
4. ABSTRACT - DANISH

*Escherichia coli* er den hyppigste årsag til bakteriæmi forårsaget af Gram negative bakterier og den overordnet hyppigste årsag til urinvejsinfektion hos mennesker. Bakterielle infektioner opstår når bakterier er i stand til at undslippe værtens immunsystem og får adgang til områder hvor de kan proliferere. At være metabolisk aktiv er nødvendigt for produktionen af virulensfaktorer, og de fleste antibiotika i klinisk anvendelse er afhængige af aktiv bakterievækst for at udøve deres fulde effekt. Imidlertid kender man lidt til den vækstdynamik der foregår under infektion i en vært (*in vivo*). Til trods for at flere teknikker til at måle bakterievækst *in vivo* er blevet afprøvet over de seneste årtier, så findes der stadig ingen ”gold standard” metode. Målet med denne PhD afhandling var at undersøge anvendelsen af molekylærbiologiske metoder til at måle tilstanden af *E. coli* kromosom-replikationsapparatet som udtryk for bakteriel vækstrate under værtsinfektion. Afhandlingen er baseret på tre artikler.

**I artikel I** demonstrerer vi anvendeligheden af bakteriel kromosom-replikationsstatus som et middel til at måle *E. coli* *in situ* vækstrate under vækst i peritoneum og i blod under eksperimentel dissemineret infektion i mus. Ved at kombinere to komplementære metoder (real-time quantitative PCR [qPCR] og fluorescensmikroskopi) til differentiel kromosomal ”origin” (*oriC*) og ”terminus” (*terC*) kopinummer-kvantificering (*ori:ter*) i fluorescensmærket *E. coli*, var vi i stand til at måle *in vivo* bakterievækstrate både på et populationsgennemsnit og på enkeltcelleniveau. I denne model demonstrerer vi: (i) at de voksende bakteriepopulationer er vedvarende heterogene (dvs. sammensat af bakterieceller der vokser ved forskellige hastigheder), (ii) at et fuldstændigt ophør af vækst ikke forekommer, og (iii) at *E. coli* ikke vokser isoleret i blod under sepsis.

**I artikel II** udvider vi metoden til at måle bakterievækstrate ud fra differentiel kromosomal ”origin” og ”terminus” kopinummer-kvantificering til at udforske dens potentiale til at forudsige antibiotikas aktivitet som en funktion af *in situ* bakterievækstrate i den samme eksperimentelle infektionsmodel. Her demonstrerer vi at aktiviteten af ceftriaxon (et β-lactam antibiotikum) og
gentamicin (et aminoglycosid antibiotikum) begge var afhængige af aktiv bakterievækst, hvorimod ciprofloxacin (et fluoroquinolon antibiotikum) var mindre følsom overfor bakterievækst in vivo. Derudover, ved at analysere ori:ter efter antibiotisk behandling kunne vi demonstrierne at ceftriaxon og gentamicin behandling medførte eliminering af fortrinsvist hurtigt voksende celler, hvorimod dette ikke var åbenlyst for ciprofloxacin.

I artikel III inkluderede vi hospitaliserede patienter med E. coli bakteriuri i et klinisk studie for at undersøge anvendelsen af differentiel kromosomal “origin” og “terminus” kopinummer-kvantificering som mål for bakteriel vækstrate i urinveje hos mennesker. Vi var i stand til at måle bakterievækstrater hos patienter både med og uden (asymptomatisk bakteriuri) symptomer på urinvejsinfektion (UVI) i op til 4 påfølgende dage. Vi observerede aktiv bakterievækst i langt de fleste urinprøver. Der var en noget højere vækstrate hos patienter med UVI (sammenlignet med patienter uden) og hos patienter med invasiv infektion (sammenlignet med patienter uden), hvilket kunne antyde at aktiv bakterievækst er en faktor der bidrager til patogenicitet. Dog vil det være nødvendigt at evaluere disse observationer i en større fremtidig kohorte.

De sammenlagte observationer beskrevet i disse artikler bidrager til en større indsigt i den bakterievækstdynamik der finder sted under infektion og understreger at den aktuelle vækstrate er af betydning for antibiotikas effekt in vivo.
5. INTRODUCTION

Bacterial infections arise when bacteria evade the host immune system and access areas, e.g. tissues or mucosal surfaces, where they can proliferate. The ability to grow and divide during the infection process, i.e. being metabolically active, is a necessity for the production of virulence factors (1, 2). Also, most antibiotics in clinical use depend upon active bacterial growth to exert their effect (3–9). However, while much is understood about the virulence factors utilised by bacteria, such as *Escherichia coli*, less is known about the growth dynamics taking place during host infection. One of the shortcomings in traditional diagnosis of bacterial infections is that bacterial presence merely is detected by growth on agar plates. This method does not provide information about the metabolic activity of the bacteria at the time of sampling. To date, there exists no gold standard method for detecting the growth rate of bacteria propagating in the host during infection. Along with a better understanding of the pathogenesis itself, insight into the bacterial growth dynamics taking place during infection could prove helpful in evaluating future antibacterial treatment strategies. The aims of this PhD thesis were to explore the use of molecular biology methods to measure the level of *E. coli* chromosome replication as readout of bacterial growth rate during host infection, and to examine the association between *in situ* bacterial growth rate and antibiotic effect.

5.1 Bacterial growth *in vivo*

The survival of bacteria depends upon nutrient availability. *Escherichia coli* is a versatile organism found predominantly in the lower intestine of warm-blooded vertebrates, but can translocate to and infect other extraintestinal sites (10). To successfully navigate and survive in these different niches the bacteria must rapidly adapt to changing environmental conditions, including nutrient availability, pH, oxygenation, competition with other residents of the host microbiota and the presence of the host immune system (10–12). Moreover, the production of virulence determinants essential for a pathogen to establish host infection is controlled by nutrient availability (1). Humans and other animals could in general be considered a good source of nutrients for bacteria, as tissues contain a large variety of nutrients, including sugars,
amino acids, iron and nitrogen-containing compounds such as ammonia and urea (1, 11).
Pathogens have evolved mechanisms to access host nutrients. These include the biosynthesis of
a wide selection of virulence factors that can kill host cells and catabolize macromolecules (1, 
11, 13, 14). However, the exact composition of nutrients and other environmental factors
affecting pathogen growth at different sites in its natural host (in vivo) remain largely unknown
(15). Also, to what extent the physical and chemical properties of the infected site change
throughout the infection process remains elusive. The capability of pathogens to induce
infection depends on sufficient growth of the pathogen to secure its establishment within the
host by colonisation or successful transmission to another host (16). Growth rate is considered
an important aspect of bacterial pathogenicity (12). To establish infection, a few bacteria having
successfully invaded the host must grow and multiply rapidly to overcome the removal of
bacteria by the host immune defences or by mechanical removal (e.g., by urination, defecation
or mucus excretion). In acute disease, rapid bacterial growth in tissues is essential for the
pathogen to produce its harmful effects before an effective host immune response arises (12, 
17). In chronic disease, however, slow growth might be beneficial for the established bacteria,
as it provokes less stimulation of the immune response and most antibacterials exert their
greatest effect on rapidly growing cells (12, 17, 18).

From fundamental laboratory studies performed several decades ago, it has been established
that the environmental conditions determine the bacterial growth rate, and that the most basic
cellular processes, including cell growth and division, are precisely coordinated with
chromosome replication to ensure accurate maintenance and inheritance of the genetic
material (19–21). In the model bacteria E. coli, the initiation of chromosome replication is
coupled to growth rate predominantly through nutrient-dependent changes in the activity and
synthesis of the initiator-protein DnaA in such a way that initiation of chromosome replication
takes place at a fixed ratio of cell mass to replication origin, called the initiation mass (19, 22, 
23). Hence, nutrient-rich growth conditions allow for short doubling-times, large cell size and
high DNA content (i.e., overlapping chromosome replication cycles) and consequently an
increase in population size, while nutrient-poor growth conditions entail long doubling times,
small cell size and low DNA content (i.e., one or no chromosome replication cycles) and consequently no or little increase in population size (20, 21).

While much is known about the genomic composition and virulence factors utilised by many pathogenic bacteria, it has remained a challenge to measure growth rates of bacteria in their natural host. Under controlled laboratory growth conditions, growth rate can readily be inferred from the linear regression derived slope of repeated measurements of culture optical density (OD) or viable bacterial counts (colony forming units [CFU]/ml) plotted as a function of time. Given aerobic, nutrient-rich and temperature optimal conditions, the model bacteria *E. coli* can grow with doubling times down to 20 minutes in a laboratory batch culture (24). These optimal growth conditions, however, do not necessarily reflect those encountered *in vivo*. Measuring bacterial growth rates during propagation *in vivo* is not as straightforward as in a laboratory setting.

### 5.1.1 Means to measure bacterial growth rate *in vivo*

Different models to quantify bacterial growth rate during in-host propagation have been proposed over the past decades. An estimation of growth rate inferred from *in vivo* bacterial count kinetics has been carried out by several investigators and measured in different bacterial species and hosts. Examples include estimates of overall growth rates of the intestinal microbiota of mice, hamsters and guinea pigs by Gibbons and Kapsimalis (25), as well as the growth rate of *E. coli* introduced into the gut of gnotobiotic mice performed both by the previously mentioned investigators (25) and by Freter et al. (26), the growth rate of experimental *Mycobacterium leprae* infection in foot pads of mice by Rees (27) and the growth rate of experimental *Haemophilus influenzae type b* during bacteraemia in rats by Rubin (28).

However, inferring bacterial growth rate directly from bacterial count kinetics is not very suitable *in vivo*, as the parallel bacterial elimination by host defence mechanisms is not taken into account. Hence, only the net result of bacterial multiplication and elimination by the host immune system is reported; the so-called net rate of change (29). It can be argued that a very rapid bacterial population density increase indeed represents a high population growth rate, despite any effect of host immune defence, whereas during slow or no population increase the
growth rate is less clear (15). The latter could reflect bacterial elimination or destruction by host defences, as well as restriction of growth inflicted by the host defences or by limiting nutritional conditions, or a combination of the above (15). Hence, true bacterial growth rate during slow or no population increase could be masked by a high kill rate by an efficient host immune defence when inferring bacterial growth rate from change in net bacterial population size. Indeed, a stationary population in vivo does not necessarily reflect cessation of bacterial growth.

To overcome the limitation of an undefined bacterial clearance or death rate, other investigators have applied experimental methods better adapted to measuring growth rate from bacterial count kinetics in vivo. Meynell (30) introduced, in the late 1950s, the quantification of dividing bacteria by genetic marker distribution, by the use of a superinfecting non-replicating phage introduced into E. coli before intravenous inoculation into mice and later recovery from the liver and spleen. Here, since the mutant phage introduced is non-replicating, it will only be passed on to one of the two daughter cells upon cell division. Hence, the fraction of the bacterial population harbouring the marker will be halved at each cell division, and from the rate at which the marker is lost, the doubling time can be estimated (30). Other investigators have applied the same principles in bacteria superinfected with a non-replicating phage. Examples include Salmonella Typhimurium infection in the spleen of mice by Maw and Meynell (31), and later by Hormaeche (32), as well as E. coli muscle infection in mice by Polk and Miles (33). Meynell and Subbaiah (29) also applied a similar approach to estimate the growth rate of S. Typhimurium during infection in the mouse gut by the use of another genetic marker: a histidine gene introduced by phage transduction in an abortively transduced state. The genetic marker of an abortive transductant does not replicate, which allows for a quantification of the proportion of cells carrying the reporter gene in the population recovered after infection compared to the ditto proportion inoculated, which would reflect the number of cell divisions having occurred during the observation period (29). Other reports by Morris Hooke et al. (34) and Sordelli et al. (35) describe the use of temperature sensitive (ts) mutants of E. coli and Pseudomonas aeruginosa used in mixture with their respective wild-type isogenic strain in peritonitis in mice (E. coli and P. aeruginosa) and in lung infection in mice (P.
In this model, the number of bacterial generations over time was deduced from the proportion of viable ts mutants (assumed not to grow and multiply at nonpermissive murine body temperature) relative to the proportion of viable wild-type cells (growing at murine body temperature). All of the models presented above (29–35), rest upon the assumption that the mutant cells are being cleared by the host at the same rate as the wild-type cells, and that murine body temperature is truly nonpermissive.

Growth rates have been estimated by others from progressive loss of specific activity of radioisotopically labelled bacteria. Baselski et al. (36) and Sigel et al. (37) applied $^{35}$SO$_4$-labelling to infer bacterial growth rates of *Vibrio cholerae* during colonisation of the upper bowel in mice from comparison between specific activity (i.e., the ratio of radioactivity to viable bacterial counts) of the inoculum and the specific activity of gut homogenates post infection. A limitation to this technique is that it cannot distinguish live from dead cells, as radiolabel can leach from both (15).

Other investigators have applied methods to quantify the ribosomal contents of bacteria. Poulsen et al. (38) inferred bacterial growth rates of *E. coli* during colonisation of the mouse gut from ribosomal content measured by quantitative single-cell in situ rRNA hybridisation with a probe specific to *E. coli* 23S rRNA. Growth rates were translated from standard curves generated during controlled growth *in vitro* correlating growth rate to ribosomal content. A similar approach was later applied by Yang et al. (39) to measure the growth rate of *P. aeruginosa* in the sputum of chronically infected cystic fibrosis (CF) patients by fluorescent in situ hybridisation with a probe specific to *P. aeruginosa* 16S rRNA. The above mentioned technique rests upon the assumption that there is a linear relationship between ribosomal content and growth rate at all growth rates *in vivo*. However, this correlation might not be valid after transition from exponential to slow growth, where the ribosomal content can become decoupled from growth rate (40).

More recently, Kopf et al. (41) and later Neubauer et al. (42, 43) demonstrated the use of stable isotope (heavy water, D$_2$O) trace incorporation into the biomass during lipid biosynthesis of
Staphylococcus aureus in the sputum of infected paediatric CF patients as a measure of metabolic activity and proxy for growth rate. One of the limitations to this approach is the necessity of incubation of the sputum sample with heavy water for trace incorporation post sample collection, which could affect the in situ metabolic state of the bacteria.

Other marker-dependent methods investigated include the use of fluorescent proteins engineered into the infecting bacteria. Helaine et al. (44) studied the growth rate of intracellular S. Typhimurium in macrophages in the murine spleen by fluorescence dilution. The infecting bacteria carried dual fluorescence reporter plasmids, and, assuming that the fluorescent proteins are distributed equally at cell division and that fluorescence is consequently halved by each generation, the average number of generations elapsed over time can be calculated from flow cytometry. Myhrvold et al. (45) applied a similar approach, called distributed cell division counting (DCDC), to estimate the growth rate of E. coli during colonisation of the mouse gut from dilution of a fluorescence label measured in the mouse faeces, and Claudi et al. (46) measured the growth rate of S. Typhimurium carrying a plasmid expressing TIMER-protein fluorescence in murine spleen homogenates by flow cytometry. A limitation to the fluorescence marker approach, is that growth rate can only be monitored up to 10 generations, after which fluorescence signalling is too weak to be accurately measured (44–46).

In recent years, as complete bacterial genome sequences have become broadly available, it has been possible to track bacterial growth from sequence tagging methods, mutation accumulation experiments and metagenomics analyses. Grant et al. (47) inferred growth rates of S. Typhimurium during invasive murine infection with various wild-type isogenic tagged strains (WITS) from mathematical modelling and relative distributions in the liver and spleen at different stages of the infection process measured by real-time quantitative PCR (qPCR). Gibson et al. (48) compiled, from the literature, estimates of the mutation rate per year (accumulation rate) of various bacteria (E. coli, P. aeruginosa, S. enterica, S. aureus and V.cholerae) in vivo and estimates of the respective mutation rate per generation in vitro from mutation accumulation experiments and whole-genome sequencing (WGS); from which an average in vivo bacterial
growth rate per year is being expressed as accumulation rate per year divided by mutation rate per generation. This method rests upon the assumption that mutation rates per generation are identical in vivo and in vitro, whereas it seems likely that in vivo mutation rates in reality are higher than those in vitro (48). Also, it provides only an average growth rate per year. Recently, Korem et al. (49) proposed a general method by which growth rates can be inferred from sequence read depth across microbial genomes. From whole genome sequencing data, they showed that metagenome read coverage patterns for several bacterial species in the human gut microbial community contain a single peak and a single trough; the former coinciding with the origin of chromosome replication (49). The technique is based on the principle that most bacteria with a single chromosome replicate their genome in an organised process where bidirectional replication forks initiate from a single, defined origin and move toward a single terminus (21, 50). During rapid growth, a large fraction of cells undergo chromosome replication and multiple replication forks are present in each cell, which results in a ratio higher than 1:1 between near-origin DNA and near-terminus DNA; hence, the peak-to-through ratio (PTR) provides a quantitative readout of the in situ bacterial growth rate (49). Analysing 583 different human metagenomic faecal samples of E. coli, Korem et al. found growth rates within the range of that observed during growth in vitro (49). Moreover, the authors demonstrated that bacterial growth rates vary among different physiological conditions; exemplified by a significant change in global gut microbiota growth rate between different dietary regimens in two healthy volunteers, by the presence of anatomical site-specific growth rates of Streptococcus salivarius and Bacteroides vulgatus inferred from existing metagenomic data sets with multiple body sites (oral cavity vs. stool) from the same individuals, and by the difference in global gut microbiota growth rate between three different species of mice (i.e., different host genetics) (49). Also, from experimental murine gut colonisation with either virulent or avirulent Citrobacter rodentium the authors demonstrated a correlation between bacterial growth rate and virulence, and from existing metagenomics data sets they demonstrated a correlation between bacterial growth rate and human disease (e.g. inflammatory bowel disease and type II diabetes correlated both with global microbiome growth rate and selected bacterial growth rates) (49). Olm et al. (51) later applied the PTR method to identify differential in situ growth rates of identical bacterial strains growing in the mouth, gut and skin of two premature infants,
and Forsyth et al. (52) applied the method to detect rapid growth rates of *E. coli* in the urine of 8 women during urinary tract infection. A limitation to the PTR method is that it is restricted to bacteria with a complete reference genome available. Brown et al. (53) extended the method described by Korem et al. to include bacterial growth rate measurements based on sequencing coverage trends for draft genomes of several bacterial species from the adult and infant human microbiota. By measuring the rate of decrease in average sequence coverage from origin to terminus of replication, the ratio between coverage at the origin and terminus of replication, respectively, can be estimated (Index of Replication [iRep]). This metric requires, however, a minimum of 75% complete genome sequence to be accurate, and was then shown to correlate to PTR (53).

The concept of inferring bacterial growth rate directly from metagenomic data (PTR or iRep) is intriguing, as it reports directly on the bacterial physiology of the bacteria without the use of an experimental marker. One of its restraints, however, is that it reports only on the population average growth rate and is unable to discern possible variations in growth rate within the bacterial population.

### 5.2 *Escherichia coli*

Most of our current knowledge on bacterial physiology and growth dynamics is based on frame work in the model organism *Escherichia coli*. It is a bacterium that is easy to isolate, grows well in the laboratory and is an important microorganism in biotechnology, being the most commonly used in the field of recombinant DNA technology (54–57).

*E. coli* is a versatile microorganism. It is a member of the bacterial family of the *Enterobacteriaceae* and is a non-sporulating Gram negative facultative anaerobe (54, 58). It inhabits the gastrointestinal tract of vertebrates as part of the commensal microbiota, and comprises the most prevalent facultative anaerobic bacteria of the human intestinal microbiota (10, 58). Human infants are colonised within a few hours after birth (10). The mucus layer lining the membranes of the large intestine, especially in the caecum and colon, acts as its niche. Here, it lives in symbiosis with the host, to whom the normal *E. coli* provides protection against
pathogen colonisation in return for a stable environment with a constant supply of nutrients and an optimum temperature of around 37°C. Glyconate from the mucus serves as the most important nutrient source (58).

Conversely, *E. coli* is also one of the most common human pathogens, with the ability to cause various intestinal and extraintestinal diseases. Different pathogenic types (defined as a group of strains of the same species causing the same disease) have been recognised, and are divided into two groups based on the type of virulence factors present and host clinical symptoms: *Enteric or Intestinal Pathogenic E. coli* and *Extraintestinal pathogenic E. coli* (ExPEC) (10, 54). The seven pathogenic types belonging to the *Enteric E. coli* group (*enteropathogenic E. coli*, *enterohaemorrhagic E. coli*, *enterotoxigenic E. coli*, *enteroaggregative E. coli*, diffusely adherent *E. coli*, *enteroinvasive E. coli* and adherent invasive *E. coli*) are associated with enteric or diarrhoeal disease, whereas the three pathogenic types belonging to the ExPEC (uropathogenic *E. coli*, neonatal meningitis *E. coli*, avian pathogenic *E. coli*) cause urinary tract infection (UTI) and neonatal meningitis, as well as extraintestinal disease in birds (10, 13, 54). *E. coli* constitutes the most prevalent causative organism in UTI in humans (approximately 80% of uncomplicated UTIs are caused by uropathogenic *E. coli* [UPEC]) and the most common invasive Gram negative isolate (10, 13, 59, 60). Moreover, *E. coli* is also found in the environment, in water, soil and sediment, where it is often used as an indicator of faecal contamination (61).

### 5.2.1 Pathogenicity and the *E. coli* genome

Commensal *E. coli* strains generally benefit the host by colonisation resistance toward other pathogenic strains. The genetic composition of commensal *E. coli* is shaped by adaptation to multiple host and environmental factors (62). These strains generally lack the virulence factors that enable the pathogenic types of *E. coli* to invade and adapt to other niches where they can cause disease in the host. The combination of virulence genes present and the level of expression of these in the different *E. coli* isolates determine their pathogenicity (14). In general, the quantity of virulence factors expressed by a strain is positively correlated to the severity of the infection it is able to cause (59). These virulence traits are frequently encoded on
mobile genetic elements that can be exchanged between strains to create new combinations of virulence (10).

The pathotypes are specific clonal types that constitute the most successful combinations of virulence factors, and they are characterised by shared antigens: O (lipopolysaccharide, LPS), H (flagellar) and K (capsular) antigens that define serogroups (O antigen only) or serotypes (O and H, and sometimes K antigens) (10). O antigens are LPS components in the cell wall and play an important role in the pathogen-host interaction, whereas H antigens are flagella mediating bacterial motility, and the K antigens make bacteria resistant to phagocytosis from host polymorphonuclear leucocytes (63–65). The virulence factors can be encoded from various mobile genetic elements, including transposons, bacteriophages, plasmids or pathogenicity islands (PAI; large genomic regions encoding one or more virulence factors). (10, 66).

Moreover, *E. coli* can be classified into four main phylogenetic groups based of combinations of the absence or presence of specific genetic markers (*chuA* and *yjaA*) and a specific DNA fragment (TspE4): A, B1, B2 and D, respectively (67). These groups differ in their ecological niche and certain growth characteristics, such as the ability to exploit different sugars and antibiotic resistance profile (67, 68). Commensal strains usually belong to groups A and B1, enteric strains to groups A, B1 and D, and extraintestinal strains to groups B2 and D, whereas strains that are able to persist in the environment usually belong to the B1 group (67).

The great variety in ecological niche, physiology and pathogenicity in *E. coli* is reflected in its highly variable genome, which, as a result of numerous horizontal gene transfer events and genome reductions, varies in both composition and size. The first complete *E. coli* genome to be sequenced was the laboratory work-horse strain K-12 MG1655, which has a relatively small size of approximately 4.64 Mb and roughly 4000 protein-encoding genes (69). For comparison, the UPEC model strain CFT073 has a genome size of approximately 5.2 Mb and over 5000 genes, out of which roughly 2000 genes belong to pathogenicity islands that are not present in the K-12 strain (66, 70, 71). A similar quantity of genes has been identified in the enterohaemorrhagic outbreak strain O157:H7, with a genome size of approximately 5.6 Mb (72). Nevertheless,
comparison of predicted proteins from the three strains has revealed that only approximately 39% of the combined set of proteins were common and derived from the so-called core-genome (10, 70–72). The remaining genome constitutes a variable gene pool, indicating high plasticity of the *E. coli* genome as a result of adaptation strategies towards different environments (58). In general, pathogenic *E. coli* strains have larger genomes than *E. coli* K-12, which partially can be explained by genetic material encoding factors that allow survival and virulence in otherwise hostile host environments (13). The availability of complete genome sequences of various pathogenic *E. coli* strains has allowed for more insight into its pathogenicity, which is often complex and multifactorial. UPEC constitute the clonal groups that are among the most prevalent in human infections, and will be described in more detail below (73).

5.2.2 Urinary tract infection

Urinary tract infection (UTI) is manifested by three clinical presentations: asymptomatic bacteriuria (ABU), lower urinary tract infection (cystitis) or upper urinary tract infection (pyelonephritis). UTIs can be further classified into: (i) uncomplicated UTIs (acute or recurrent cystitis or pyelonephritis in non-pregnant, premenopausal women without comorbidities or urinary tract abnormalities), (ii) complicated UTIs (UTI in pregnant women, men, patients with urinary tract abnormalities, permanent urinary catheter, renal disease, and/or other disease related to immune incompetence (e.g. diabetes)), (iii) recurrent UTIs (minimum 3 episodes in 12 months or 2 episodes in 6 months of recurrent uncomplicated or complicated UTI), (iv) catheter-associated UTIs (UTI in patients with urinary catheter present or having undergone catheterisation within 48 hours) and (v) urosepsis (deleterious systemic host response to bacteraemia originating from the urinary tract and/or male genital organs) (74, 75).

ABU is characterised by the presence of significant quantities (often defined as $>10^4$ CFU/ml) bacteria in the urine without any clinical signs or symptoms of infection (76, 77). The long-term consequence of ABU is dependent upon the human population. In healthy individuals, ABU may prove beneficial to the host as it has been observed to protect against infection from other more virulent bacteria (76), whereas asymptomatic bacteriuria in pregnancy left untreated is
associated with pyelonephritis, preterm labour and low birth weight (78). Cystitis is characterised by symptoms of dysuria, urgency and frequent urination and possibly suprapubic pain, and 75 – 85 percent of acute, uncomplicated cases of cystitis are caused by *E. coli* (79). Pyelonephritis is characterised by fever, general malaise, and bi- or unilateral flank pain, with or without accompanying symptoms of cystitis (13). It represents the most severe form of UTI, and may progress into urosepsis and is associated with significant morbidity and mortality (80, 81). *E. coli* is the most prevalent causative organism (73, 81).

Pathogenesis of UTI caused by UPEC is complex and under the influence of both host biological and behavioural factors, as well as the virulence traits of the infecting strain. Bacterial growth in normal human urine is prevented by its inherent antibacterial activity, which is correlated predominantly to a high concentration of urea, low pH and a high osmolality (82). Dilution and removal of bacteria by urination comprises another important mechanical host defence mechanism against urinary tract infection (83). Nonetheless, it has been argued that urine provides excellent opportunities for growth of the bacteria that cause urinary tract infection (82), likely due to genetic adaptation for growth in this niche. UPEC, like other ExPEC, do not have a unique virulence profile. However, they generally do possess a combination of virulence factors that allow them to cause host infection (84–86). The arsenal of virulence factors identified in UPEC include fimbrial adhesins, flagella, toxins, auto-transporter proteins and iron-acquisition systems (13, 87). Most ExPEC, including UPEC, have the ability to reside within the intestinal tract without causing harm to the host and may disseminate to and colonise other anatomical sites where they cause disease (60). Up to 95 % of all UTIs develop by ascension of faecal bacteria from the host’s intestinal microbiota having colonised the periurethral area (13). Access to the bladder is achieved via the distal urethra and introitus, and in some cases progression to the kidneys via the ureters occurs. Women are at higher risk of UTI than men, predominantly due to a short urethra and short distance between external urethral orifice and anus. UTI may arise from bacteraemia originating from other anatomical sites via the kidneys in extremely rare cases (13).
Fimbrial adhesins are surface components important for successful colonisation by mediating adherence to host cells, which constitutes the first critical step in establishing an infection (87). Fimbrial adhesins associated with UPEC pathogenicity include Type I fimbria, P fimbriae, Dr adhesins and F1C/S fimbriae, out of which Type I fimbriae is the best characterised (10, 13, 59, 87–90). Type I fimbriae bind to proteins such as uroplakins that are abundant in the bladder via the FimH adhesin (88, 89). Besides its role as a mediator of adherence, Type I fimbriae are responsible for activation of immune response and host cell invasion, which allows for the establishment of intracellular bacterial communities (IBCs) (13, 91). IBCs have been observed both in murine models of UTI and in voided uroepithelial cells collected from patients (92). This intracellular niche is beneficial, as the UPEC appear to form biofilm-like structures and remain protected from both components of the host immune response and antibiotics (92, 93). It has been proposed that recurrent UTI could result from reactivation of a few remaining IBCs (13).

5.3 The cell cycle of E. coli

Prokaryotes like E. coli reproduce by binary fission. To survive and ensure genome integrity between generations it is necessary to make identical copies of the chromosomal DNA before cell division. The duplication of DNA needs to be highly accurate and timely regulated in the cell cycle. The growth rate of E. coli is controlled by the chemical and physiological composition of its growth environment, predominantly by nutrient availability, allowing for a wide range of doubling times (20, 21). E. coli can achieve doubling times of ~ 20 minutes when growth conditions are optimal, including a growth temperature of approximately 37°C and access to a good carbon source, such as e.g. glucose, and building blocks essential for protein synthesis (20, 24, 94, 95). Contrarily, given disadvantageous growth conditions doubling times may be up to several hours. Part of the success for E. coli to survive in nature is its ability to rapidly adapt to environmental changes by adjusting growth rate accordingly (21, 96, 97). Any change in growth rate must go hand in hand with changes in the cell cycle to ensure that cell division remains coordinated with mass doubling and chromosome replication (21, 97). The cell size is equivalent to the total biosynthesis per division cycle, and adaptation to a new physiological state results in a change in cell size (19, 98, 99). Hence, the total biosynthesis is uniquely
determined by the growth conditions. When cells are in balanced growth the cell size and all cellular components double at the same rate (100).

The bacterial cell cycle or division cycle is defined by the series of events taking place in the cell prior to cell division and is divided into three stages: the period between cell division and the initiation of chromosome replication (the B period), the period of chromosome replication (the C period) and the period between chromosome replication termination and completion of cell division (the D period) (101). Both the C and the D period remains largely invariable: the C period, corresponding to the fixed chain elongation time (21), lasts approximately 40 minutes at doubling times < 60 minutes due to a relatively fixed rate of speed of the DNA polymerase (102) and the D period lasting approximately 20 minutes at moderate to fast growth rates (103). Hence, increase in doubling time primarily relates to increase in B period (103).

Contrarily, to achieve doubling times lower than the time required for replication of the full chromosome, multiple chromosome replications must be initiated within the same division cycle (21). Hence, during rapid bacterial growth overlapping rounds of chromosome replication occur, and, during slow bacterial growth, intervals without chromosome replication occur (Figure 1); from which we can conclude that initiation of chromosome replication is the critical parameter for bacterial growth control (103).

*E. coli*, like most other bacteria, harbours a single circular chromosome of double-stranded DNA consisting of two strands of nucleotides bound together by hydrogen bonds in complementary base pairs to form a double-helix (103). DNA replication occurs in a semi-conservative manner, where each of the parent strands serves as template for a new double-strand (104). Chromosome replication is highly conserved and proceeds in three phases: initiation, elongation and termination, which will be explained in more detail below.
Figure 1 The replication cycle of *E. coli* in slowly and rapidly growing cells. The chromosomal replication cycle and the cell division cycle are shown. During slow growth (exemplified by a mass doubling time [τ] of 80 min) cell division occurs after completion of chromosome replication. During rapid growth (exemplified by a τ of 35 or 25 min) initiation of replication occurs in synchrony at each origin of replication (*oriC*) on the partially replicated chromosomes within the cell, and cell division occurs after the completion of the previous round of replication. Origin of replication (*oriC*) is designated by closed green circles and the terminus of replication (*terC*) by closed red circles. Figure adapted from (105).

### 5.3.1 Initiation of chromosome replication at *oriC*

Initiation of chromosome replication is highly regulated and takes place at a single, unique DNA sequence called the origin of replication (*oriC*) (106–109). The *oriC* is a well-defined 245 bp sequence located between the *gidA* and *mioC* genes. It has two distinctive functional domains:
the duplex-unwinding element (DUE) containing three AT-rich repeats of a 13 bp sequence (termed L [left], M [middle] and R [right]), and the DnaA-oligomerisation region (DOR) containing specific binding sites for both positive and negative regulators of initiation; IHF and Fis, respectively (107). Moreover, the DOR is subdivided into sub-regions with different directionality (left-half, middle and right-half). Within these, there are 12 DnaA-binding sites (DnaA boxes) that can bind DnaA with different affinity (Figure 2) (106–108).

**Figure 2** The circular *E. coli* chromosome (top) with the location of oriC and terC indicated, and the basic structure of oriC (bottom). The 245 bp oriC region is located between genes gidA and mioC, with the duplex-unwinding element (DUE) to the left and DnaA oligomerisation region (DOR) to the right. The AT-rich 13-mer sequences (left [L], middle [M] and right [R]) of the DUE region are indicated with red arrows. The DnaA-binding sites of the DOR and their directionality are designated by light blue (low-affinity), green (moderate affinity) and dark blue (high affinity) triangles. The IHF-binding site (IBS) is designated by a yellow square. Figure adapted from (107).

DnaA is the rate-limiting initiator-protein responsible for double-strand opening at the DUE region and for the recruitment of replisome components; that is, the numerous proteins working in concert as a complex replication machine (103, 110–113). The DnaA protein is a highly conserved AAA⁺ protein (ATPases Associated with diverse Activities) that can bind both ATP and ADP with similar high affinities (50, 103). The high affinity DnaA boxes (R1, R4 and R2)
can bind both ATP and ADP associated DnaA (DnaA\textsubscript{ATP} and DnaA\textsubscript{ADP}), whereas the multiple low affinity DnaA boxes (R5/M, I1, I2, I3, C1, C2, C3, τ1 and τ2) can only bind ATP associated DnaA (DnaA\textsubscript{ATP}) (106–108). Only DnaA\textsubscript{ATP} bound to DnaA boxes in the oriC can initiate chromosome replication by causing an unwinding of the AT-rich region of oriC (106). An assembly of approximately 20 molecules of the initiator-protein in its active form (DnaA\textsubscript{ATP}) is required for initiation of replication (114), which occurs when oriC-bound DnaA\textsubscript{ATP} oligomers displace the negative regulator Fis from its binding site, allowing for IHF to bind to its site and bend the DNA 180° and thereby DnaA to form a cross-strand interaction that induces unwinding of DNA at the DUE region (108). DnaA-oriC-complex induced double-strand opening causes the formation of a so-called replication bubble and allows for a number of proteins to assemble and bind to each of their single-strand template, forming replication forks moving along the respective template strand in a 5’ to a 3’ direction (103). The replication machine is comprised of multiple replication enzymes including DNA polymerase, circular sliding clamp and clamp loader, helicase, primase and single-strand binding proteins (SSB), and is referred to as the replisome (103, 115).

DnaA interacts with the DNA helicase complex (a protein complex formed by the DNA helicase, DnaB, and the helicase loader, DnaC) that bind to each of the two exposed single DNA strands at either side of the replication bubble (106). After loading of the DnaB onto the single-stranded DNA, DnaC dissociates from DnaB, after which the helicase actively translocate along single-stranded DNA and unwinds the DNA helix at the front of the replication fork using energy from ATP hydrolysis (107, 116). DNA polymerase III holoenzyme is loaded onto the template strand by a sliding clamp and clamp loader complex and synthesises new DNA by repeated addition of single nucleotides. DNA polymerase cannot, however, initiate a new chain of nucleotides without an existing RNA sequence. This is introduced by a DNA-dependent RNA polymerase, the DNA primase DnaG, which transiently binds to the DnaB and synthesises a short RNA sequence, a primer, on the template strand (Figure 3) (103, 106).
**Figure 3** Initiation of replication in *E. coli*. At the initiation of chromosome replication double-stranded DNA within oriC is opened by the binding of the initiation protein DnaA, generating single-stranded DNA available for replication. The primosome (a complex comprising the DNA helicase [DnaB] and the DNA primase [DnaG]) and the DNA Polymerase III holoenzyme [Pol III HE] complex are assembled around the single-stranded DNA and replicate bidirectionally around the circular chromosome. Pol III is a multi-subunit enzyme consisting of three key components: the core polymerases (each made up of three subunits: α, ε, and θ) which provide polymerase and proof-reading activities, the β₂-sliding clamps (made up of two β-subunits) which enable progressive DNA-synthesis, and a clamp loader complex (made up of a total of seven subunits: three τ subunits, a δ-subunit, a δ'-subunit, a χ-subunit and a ψ-subunit, collectively called τ3δδ'χψ) which loads and unloads the sliding clamps onto the DNA, as well as tethering the three core polymerases. Figure from (117).

### 5.3.2 Elongation

During elongation, the DNA helicase (DnaB) on either side of the replication bubble formed at oriC move along the respective template strand in a 5’ to 3’ direction, establishing two replication forks moving away from each other as replication proceeds; a phenomenon termed bidirectional replication (118). The two strands at the replication fork are each being replicated by their own DNA polymerase III holoenzyme, held in place by the sliding clamp and working together in concert with the helicase (115). The DNA polymerase repeatedly adds single nucleotides with bases complementary to those of the template strand. As the DNA double-strand is antiparallel with their respective 5’ and 3’ ends oriented in opposite directions, and the DNA polymerase III only adds nucleotides in the 3’-hydroxyl end of the RNA primer, the two strands are being replicated in opposite directions (103). The leading strand has 5’ – 3’ polarity.
and is continuously replicated in the direction of the replication fork, while the lagging strand has 3’–5’ polarity and is discontinuously replicated in the opposite direction by short DNA sequences (approximately 1–2 kb) called Okazaki fragments, which are later joined together by DNA ligase (119). The DNA polymerase associated with either template strand work together in concert at a constant rate of approximately 1 kb/s from initiation to termination (116, 120–122). Single-strand binding proteins (SSB) cover the newly exposed single-strand DNA at the replication fork and protect it from being cleaved by nucleases and facilitates elongation of DNA polymerase on single-stranded DNA (122).

### 5.3.3 Termination of replication at terC

While the details of initiation and elongation of chromosome replication are established, less is known about replication termination. Termination occurs at a site located 180° opposite the oriC, namely the terminus of replication (terC). Here, the two replication forks, having moved bidirectionally from the origin, eventually converge and the replication enzymes disassemble, leaving behind two new continuous double-stranded chromosomes (122). Upon replication fork encounter, the two completed chromosomes are interlinked, which needs to be resolved before segregation into individual daughter cells. This is carried out predominantly by Topoisomerase IV, one of the two type II topoisomerases (Topoisomerase IV and DNA gyrase) in E. coli (122, 123). Cell division is mediated by a large protein complex called the divisome (124). Synthesis of a mid-cell septum at the end of the cell cycle creates two new cells upon cell division. While most of the divisome proteins have been identified, the underlying mechanisms of interaction remain unclear.

### 5.3.4 Multifork replication and its regulation

The C period is limited by the fixed velocity of the DNA Polymerase III holoenzyme. To some extent, this limitation is circumvented by dichotomous chromosome replication (i.e. two forks replicating from the same unique origin and meeting halfway around the chromosome to form two new fully replicated chromosomes). E. coli has a genome size of approximately 5 Mb (K-12 strain MG1655: 4.64 Mb) (69). Consequently, replicating a chromosome of this size by
polymerisation at the speed of effectively \( \sim 2 \times 1000 \) bases per second would take approximately 40 minutes (C period). Nevertheless, *E. coli* exert doubling times below this limit; with a minimal doubling time of about 20 minutes. To achieve generation times lower than the replication time, multifork replication occur within a single cell as a result of overlapping replication cycles (94, 103). This results in newborn cells having more than one oriC at birth. The number of origins per cell is given by \( 2^n \), where \( n \) is the number of overlapping cell cycles before division (125). The power of 2 implies that the oriC copy number doubles with every round of replication initiation. For initiation of replication at multiple origins to be carried out successfully, initiation of replication must be synchronised and highly regulated, allowing for initiation at all origins to occur once and only once per cell cycle. Two key properties ensure that the frequency of chromosome replication matches the growth rate in *E. coli*: initiation mass and initiation synchrony (19, 105, 125, 126).

Chromosome replication is accurately coordinated with cell growth by a defined ratio of cell mass to replication origin at initiation of replication: the so-called initiation mass (19). Donachie found that the initiation mass was constant in *E. coli* growing at doubling times \( \leq 60 \) minutes; meaning that although faster growing cells were larger and had more DNA, the origin-to-mass ratio was similar to that of the smaller, slower growing cells (19). This has later been reproduced by other investigators who confirmed the consistency of initiation mass under various controlled growth conditions, including extensive growth inhibition (98). The DnaA protein is the link between initiation of replication and cell growth (22, 109, 127), as the initiation onset is determined by a cell-cycle dependent peak in the ratio of DnaA\(^{\text{ATP}}\) to DnaA\(^{\text{ADP}}\) (105, 128).

Several regulatory mechanisms are involved in the maintenance of initiation synchrony, allowing multiple initiations to occur in synchrony from \( 2^n \) \((n = 1, 2, 3)\) origins per cell once and only once per doubling time (125, 129). Premature initiations of replication can be lethal to the cell (130). Regulation of chromosome replication is focused around initiation of replication and target DnaA or oriC. Firstly, the dnaA gene is autoregulated, with the active form of the protein acting as an efficient repressor (131, 132). Moreover, there are three key regulatory
mechanisms that ensure coordinated initiation and prevent untimely reinitiation by regulating the activity of DnaA on oriC. These include sequestration by the SeqA protein, DnaA titration and DnaA inactivation.

There is a high frequency of GATC sequences in both the oriC and the dnaA promoter (108). The Dam methylase recognises these sequences and methylates the A residues (133). As for oriC, this feature serves to distinguish new origins from old origins, as the newly replicated origin will be methylated only on one strand (hemimethylated) and the old ones on both strands (129). The hemimethylated GATC sequences are recognized and sequestered by the SeqA protein (134–136). As a result, reinitiation is prevented both by blocking DnaA access to the newly replicated origin and by inhibiting new synthesis of DnaA for about 1/3 of the cell cycle (130, 134).

DnaA titration involves the binding of DnaA to chromosomal sites outside the oriC. There are a large number of DnaA-binding sites around the chromosome that are not involved in replication initiation (110). As these are being duplicated during chromosome replication they contribute to the titration of DnaA away from oriC (105). The datA site is situated roughly 500 kb from oriC and is duplicated shortly after initiation. With its high-affinity cluster of DnaA boxes and close proximity to oriC, this site has been believed to be the main titration site (137, 138). However, in recent years the role of datA has been re-evaluated and its main function has been demonstrated to associate with the hydrolysis of initiation-active DnaA^{ATP} to initiation-inactive DnaA^{ADP}, resulting in a lowering of the quantity of DnaA^{ATP} post initiation (139). This function is termed DDAH (datA-dependent DnaA-ATP hydrolysis).

Another essential regulatory process involved with the reduction of DnaA^{ATP} post initiation is the replication-coupled feedback system termed RIDA (regulatory inactivation of DnaA) (105, 126, 130, 139). This process involves the hydrolysis of initiation-active DnaA^{ATP} to initiation-inactive DnaA^{ADP} by the DnaA homologous protein Hda in complex with the DNA-loaded β-clamps (128, 139, 140).
At later cell cycle stages \( \text{DnaA}^{\text{ATP}} \) is regenerated to allow for novel initiation of replication at the origin. Rejuvenation of \( \text{DnaA}^{\text{ADP}} \) to \( \text{DnaA}^{\text{ATP}} \) occurs by nucleotide exchange, a reaction that is mediated predominantly by two intergenic chromosomal sites termed DARS1 and DARS2 (DnaA-reactivating sequence) (140). These sequences are located halfway between the \( \text{oriC} \) and \( \text{terC} \) at either side of the \( \text{oriC} \), and both have clusters of DnaA boxes (140, 141). DARS facilitates the release of ADP from DnaA when DnaA\(^{\text{ADP}}\) is associated with DARS. The resultant apo-DnaA is released from DARS and is subsequently likely to bind to ATP (which is approximately ten times more abundant in the cell than ADP), allowing for reactivation of DnaA (105).

Together, these mechanisms limit time window when DnaA\(^{\text{ATP}}\) is available for replication initiation at \( \text{oriC} \) to once and only once per cell cycle and prevents untimely re-initiation of replication.

Based on extensive knowledge on \( E. \text{coli} \) chromosome replication and its precise coordination with cell growth and division, a quantitative readout of bacterial growth rate can be inferred from measuring the proportion of DNA copy numbers near the origin of replication to those near the terminus of replication (i.e., \( \text{ori:ter} \)) (142). An \( \text{ori:ter} \) ratio of 1:1 indicates no ongoing chromosome replication, whereas an \( \text{ori:ter} \) ratio above 1 indicates active growth. As an increase in growth rate is achieved by synchronously initiated overlapping chromosome replication cycles, an \( \text{ori:ter} \) ratio above 2 is indicative of multifork replication. In this PhD project, I have extended this basic concept of \( E. \text{coli} \) physiology into testing its value in inferring growth rate during infection in an experimental mouse model and during infection or colonisation of the human urinary tract.
6. PAPER I
Chromosome replication as a measure of bacterial growth rate during *Escherichia coli* infection in the mouse peritonitis model

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The efficacy of most antibiotics is dependent on active bacterial growth, yet little is known about the growth dynamics during infection. Therefore, means to measure in-host bacterial growth rate is of importance. Here, we use chromosome replication as readout for *in situ* bacterial growth rate during infection; obtained from a single biological specimen. We have applied two independent methods: quantitative PCR (qPCR) and fluorescence microscopy, to quantify the level of chromosome replication present during *Escherichia coli* propagation in the mouse peritonitis model. We find that the methods complement each other and allow for quantification of growth rate, both on a population average and on a single-cell level. We demonstrate the presence of heterogeneous growth rates within bacterial populations propagating during infection. Also, no growth cessation was observed during the apparent stationary phase *in vivo*, and, by comparison of growth dynamics at different anatomical sites, we demonstrate that *E. coli* is unlikely to grow independently intravascularly. These findings provide novel insight into bacterial growth during host infection, and underscore the importance of pinpointing the primary site of infection in sepsis of unknown origin and ensuring antibiotic availability at this site.

The interplay between bacterial growth and chromosome replication has been studied extensively under laboratory conditions for decades¹⁻⁴. Until recent years, the detection of chromosome replication status in bacteria propagating in their natural host (*in vivo*) had received scant attention. Bacterial growth *in vivo* has generally been assessed by viable bacterial counts (colony forming units (CFU)/ml) plotted as a function of time. In a batch culture with a finite amount of nutrients, this curve is divided into well-defined growth phases⁷. However, bacterial growth dynamics *in vivo* are complex; growth rate is not solely a function of nutrient availability, but also of host immune-mediated clearance⁸⁻¹¹. Applying bacterial count measurements to deduce an absolute bacterial growth rate *in vivo* is therefore not adequate, as this merely reflects the net change in bacterial population size. Neither does it reflect the level of growth rate heterogeneity within the population. To circumvent some of these limitations, recently published studies have explored the use of chromosome replication status as readout for bacterial growth rate in environmental and human microbiomes and found good correlation between the two¹²⁻¹⁴. However, this was achieved by whole genome sequencing, a method that besides being expensive and time-consuming, only provides a population average readout and fails to provide information on any possible differential growth rates within the population.

*Escherichia coli*, like many other bacteria, possesses one circular chromosome. DNA replication is initiated from a single origin of replication (oriC) and carried out bidirectionally, terminating in the opposite replication terminus region (terC)¹¹⁻¹⁶. Coordination between chromosome replication and cell division ensures that the two identical daughter cells accommodate at least one fully replicated chromosome¹⁷⁻¹⁸. Initiation of replication takes place at a fixed cell mass per origin: the initiation mass³,¹⁹⁻²². Under favourable growth conditions multiple rounds of synchronously initiated replications per division cycle can occur⁴,²³. This allows for faster growth rates, with daughter cells carrying chromosomes with overlapping rounds of replication, i.e. more than one number of oriC copies (2ⁿ, n = 1, 2, 3), at birth. Due to the consistency of the initiation mass, rapidly growing cells harbouring overlapping rounds of replication have larger cell mass proportional to the number of oriC copies. The

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growth rate of the population is reflected by the copy number ratio of oriC to terC (ori:ter), with an ori:ter of 1 representing non-replicating cells carrying a complete set of chromosome(s) and an ori:ter > 1 representing cells with ongoing chromosome replication (growing cells). Theoretically, this correlation can be expressed as $\text{ori:ter} = 2^{C/\tau}$ (1), where $C$ is the chromosome replication time ($C$-period) and $\tau$ is the mass doubling time, and thus $\tau = \frac{\log(\text{ori:ter})}{2}$. However, such an unambiguous measure can only be inferred from a population of bacterial cells propagating at balanced growth rates, and only at $\tau < \text{approximately 60 minutes}$, where $C$ has been shown to be constant.

Here, we applied quantitative PCR (qPCR), an inexpensive and easily accessible method, to provide population average measurements of bacterial growth rates during infection in vivo, combined with chromosomal fluorescent marker frequency analysis for quantification of bacterial growth at a single-cell level. The goal was to determine variation in in situ bacterial growth rates at various anatomical sites, which would add to our current understanding of growth dynamics during infection. We hypothesized that growth dynamics within and outside the primary site of infection would be different. Furthermore, the detection of an in situ bacterial growth rate obtained from one single specimen could form the basis for refining future antibacterial strategies, knowing that the efficacy of most antibiotics in clinical use is related to active bacterial growth.

Results

Two methods for detection of ori:ter were tested in vivo in an experimental murine infection model; qPCR, where the ori:terqPCR was given as a population mean, and fluorescence microscopy, where the ori:termic was deduced from direct analysis of single fluorescently labelled live bacterial cells (exemplified in Fig. 1, where oriC loci are displayed in green (GFP labelling) and terC loci are displayed in red (mCherry labelling)). In ori:terqPCR, the ‘oriC copy number was inferred from a site located immediately to the left of oriC, and the ‘terC copy number’ was inferred from a site located in close proximity to terC. We applied these methods, in combination with bacterial counts, to reveal fundamental bacterial growth dynamics at various anatomical sites during infection. Furthermore, the correlation between cell length ($\mu$m) (measured by microscopy and used as a proxy for cell mass) and oriC/cell was measured to investigate initiation mass consistency during infection in vivo. As proof-of-concept, the same methods were tested and validated in vitro, using the same model infective organism; Escherichia coli ATCC® 25922™.

Bacterial growth in vitro

Bacteria grown in vitro in Lysogeny Broth (LB) batch cultures were used to validate the methods applied in the in vivo experiments. Here, stationary phase bacterial cells were diluted in fresh media and allowed to grow with repeated sample collections at time points 2, 4, 6, 8 and 10 hours of incubation. As a complete cessation of growth was observed at 10 hours of incubation, the in vitro experiments were not extended beyond this time point. We tested the wild-type strain (ATCC 25922) alongside a genetically modified derivative of the strain, with chromosomally incorporated fluorescent oriC and terC labels (ALO 4783). This was done to ensure that transgene insertion would not affect growth dynamics of the derivative strain, which was applied in the in vivo infection model.

Growth curves from in vitro studies are presented as bacterial counts as a function of time in Fig. 2a, and as OD_{600} values as a function of time in Fig. 2b, respectively. Exponential growth was observed between the initial sampling time point and approximately 6 hours of incubation, after which growth progressively ceased towards stationary phase. The mass doubling time ($\tau$) was computed from equation (2) as (median (IQR)) 23.4 (21.5–29.9) minutes (assuming a constant $C$ period of 40 minutes). Inferring $\tau$ from ori:ter and $\tau = \frac{\log(\text{ori:ter})}{2}$ (2). At 8 and 10 hours of incubation, the correlation between cell length ($\mu$m) (measured by microscopy and used as a proxy for cell mass) and oriC/cell was measured to investigate initiation mass consistency during infection in vivo as proof-of-concept.

The two methods for detection of chromosome replication status (ori:ter_qPCR and ori:termic) were in good agreement (Supplementary Fig. S1). ori:ter_qPCR and ori:termic followed growth rate (Fig. 2a, Supplementary Table S1). We observed a rapid increase in ori:ter during the first 4 hours, followed by a gradual decline as the cells stopped growing by 8 hours; remaining unchanged at 10 hours of incubation. In accordance with these findings, single-cell fluorescence microscopy analysis of oriC/cell and cell size ($\mu$m) confirmed a shift from a homogenous bacterial population dominated by non-growing, small cells at 0 hours (i.e. the inoculum; oriC/cell ~1), to a heterogeneous population dominated by bacterial cells with ongoing chromosome replications at various levels (oriC/cell ≥ 2) and various cell sizes at 2 hours of incubation (Fig. 3). Starting from 4 hours of incubation, a tendency towards more growth homogeneity was observed. At this time point, an oriC/cell of 4 was observed in approximately 80 percent of the population, reflecting close to balanced growth (Fig. 3). Here, criteria for inferring mass doubling time ($\tau$) from ori:ter were met, and $\tau$ was computed from equation (2) as (median (IQR)) 23.4 (21.5–29.9) minutes (assuming a constant $C$ period of 40 minutes). Almost no overlapping rounds of replication were observed starting from 6 hours of incubation (oriC/cell ≤ 2). At 8 and 10 hours of incubation homogenous populations of small bacterial cells harbouring predominantly one copy of oriC/cell were observed (Fig. 3). The correlation between oriC/cell and cell size ($\mu$m) was very strong ($r = 0.92, p < 0.01$) (Fig. 2c).

A slow-down of growth was initiated by 4 and 6 hours of incubation, during which period we observed a decrease in both oriC/cell and ori:ter, manifesting cessation of chromosome replication (Fig. 2a,c). As expected, cell division proceeded for some time longer, as demonstrated both by the bacterial count increase and cell size decrease (Fig. 2a,c). Eventually, no new initiations of chromosome replication took place during stationary phase (8–10 hours of incubation). Inferring $\tau$ from ori:ter for time points later than 4 hours of incubation would be deceptive, given that the growth was too slow (i.e. the criteria of $\tau < 60$ minutes was not met).

We can conclude that, when these bacterial cells were growing fast the ori:ter and oriC/cell ratios were high and cell sizes were large, and when cells were growing slowly the ori:ter and oriC/cell ratios were low and cell sizes were small. Our findings are consistent with the existence of a fixed initiation mass at all in vitro growth rates, exemplified by the good correlation between oriC/cell and cell length (Fig. 2c). These data are consistent with previous studies that have shown correlation between ori:ter and initiation mass in E. coli K-12 strain MG1655 under various growth conditions in vitro. At mid-exponential phase (4 hours of incubation; Fig. 2a) a mass doubling
time ($\tau$) could be inferred from ori:ter and was similar to that inferred from OD$_{600}$ measurements; approximately 23 and 25 minutes, respectively.

**Bacterial growth in vivo: mouse peritonitis model.** Bacterial growth dynamics at separate anatomical sites during widespread bacterial infection were evaluated in the model of lethal peritonitis in mice, aiming both to validate the ori:ter method for use in vivo, and to determine any potential growth rate differences within and outside the primary site of infection.

An intraperitoneal (i.p.) dose of $1 \times 10^6$ CFU/ml stationary phase bacteria (ALO 4783) resulted in the establishment of sepsis in a total of 51/51 animals within 2 hours of infection. Samples (peritoneal lavage fluid (PLF), blood, spleen and kidneys) were collected in biological triplicates at time points 2, 4, 6, 8 and 10 hours of infection. After 10 hours of infection animals demonstrated signs of distress to such an extent that criteria for euthanasia were met. Thus, this constituted the final possible sample collection time point in this model. Bacterial DNA and live bacterial cells were successfully isolated from PLF and blood for qPCR and microscopy analysis, respectively. Unfortunately, we were unable to adequately isolate bacterial DNA or live bacterial cells for the above mentioned analyses from spleen and kidneys. Consequently, the PLF and blood bacterial populations constituted representative populations of bacteria growing within and outside of the primary site of infection, respectively. It has previously been hypothesised that bloodstream bacterial populations represent a passive ‘spill-over’ of bacteria from the primary site of infection, rather than independently growing populations.$^{30}$ Using chromosome replication as readout for *in situ* bacterial growth rate, we aimed at improving insight into whether these populations truly differ or not.

Pooled bacterial count data from repeated *in vivo* experiments demonstrated that the net size of the bacterial populations found outside the primary site of infection (i.e. bloodstream, spleen or kidneys) increased in parallel with the bacterial population at the primary site of infection (i.e. peritoneum) (Fig. 4a). For all collected specimens (PLF, blood, spleen and kidneys) a pronounced increase in bacterial count was observed from 0 to 8 hours of infection, indicating bacterial growth. There was no apparent lag phase, however, this cannot be ruled out since...
no measurements were made prior to 2 hours of infection. Linear regression analysis derived slopes ($\beta$) for growth in blood, spleen and kidneys revealed no significant difference from growth in PLF ($p > 0.05$), whereas the blood, spleen and kidney bacterial counts differed significantly from the PLF bacterial count ($p < 0.0001$); meaning that bacterial population sizes outside the PLF were smaller, yet increasing in parallel with the PLF bacterial population (Fig. 4a). Also, attention should be drawn to the fact that the bacterial count found at the primary site of infection was under-estimated. The PLF bacterial count was in reality substantially higher than reported, taken into consideration the dilution factor from the required addition of lavage fluid during harvesting (see Methods).

From 8 to 10 hours of infection the net bacterial count was stagnant (Fig. 4a).

The maximum $\text{oriC}_{\text{PCR}}$ (mean (SD)) measured in vivo (2.50 (0.36) in PLF) was lower than the maximum $\text{oriC}_{\text{PCR}}$ measured in vitro (3.13 (0.55)), accounting for an overall slower growth in vivo (Figs 4b and 2a, Supplementary Table S1). Nevertheless, $\text{oriC}$ followed overall growth rate in vivo (Fig. 4b, Supplementary Table S1). In the PLF $\text{oriC}_{\text{PCR}}$ increased rapidly during the first 2 hours, followed by a slight decline towards unchanged levels at 4 and 6 hours of infection. After 6 hours of infection a gradual decline towards an $\text{oriC}_{\text{PCR}}$ approaching 1 was observed (Fig. 4b, Supplementary Table S1). In the blood, $\text{oriC}_{\text{PCR}}$ did not change between 4 and 8 hours. However, no significant difference ($p > 0.05$) in $\text{oriC}_{\text{PCR}}$ were found between PLF and blood, when compared both overall by linear regression analysis and time-point by time-point (2 to 10 hours of infection) (Fig. 4b).

**Figure 2.** Bacterial growth in LB batch cultures (*in vitro*). (a) Bacterial growth measured as bacterial counts (log$_{10}$ CFU/ml), $\text{oriC}_{\text{PCR}}$ and $\text{oriC}_{\text{inc}}$. ATCC 25922 and ALO 4783 were tested in parallel and showed similar growth; hence, results from both strains are pooled in the data sets. Bland-Altman agreement between $\text{oriC}_{\text{PCR}}$ and $\text{oriC}_{\text{inc}}$ was good (Supplementary Fig. S1). 6 h, $n = 12$; all other time points, $n = 6$. (b) Comparisons of ATCC 25922 and ALO 4783 grown in LB batch cultures (*in vitro*) revealed no growth retardation due to transgene insertions. Growth measured as OD$_{600}$. Both strains exerted doubling times of 25 minutes during exponential growth. (c) Bacterial cell length (µm) (used as proxy for cell mass) correlate with $\text{oriC/cell}$. Data represent pooled microscopically detected cells (ALO 4783). $n = 500$ at all time points, with the exception of 2 h, where $n = 147$. Data are presented as mean ± SD. Dotted line represents the limit of detection for bacterial count measurements. Time (h) represents hours of incubation; inoculum is presented as 0 h. $r$: Pearson’s correlation coefficient.
There was no significant difference ($p > 0.05$) in oriC/cell or cell size (µm) between PLF and blood bacterial populations, compared at 6, 8 and 10 hours of infection (Fig. 4c). At 2 and 4 hours of infection, the total number of microscopically detected live bacterial cells from the blood was too low. Hence, these data are not included in statistical analyses or illustrations (Supplementary Table S1, Figs 4c and 5). oriC/cell and cell size (µm) data

**Figure 3.** Relative frequency distributions (%) of pooled microscopically detected bacterial cells (ALO 4783) from growth in LB batch cultures (*in vitro*); cell length (µm) (left panel) and oriC/cell (right panel). $n = 500$ at each time point, with the exception of 2 h, where $n = 147$. Time (h) represents hours of incubation. For oriC/cell histograms, note that data are presented as detected by automated foci quantification, which is subject to the risk of underestimation due to co-localising oriC. Hence, it is likely that 3 detected foci truly represent 4 foci and that >4 detected foci truly represent 8 foci (see Methods).
Figure 4. Bacterial growth (ALO 4783) in the mouse peritonitis model (in vivo). (a) Bacterial growth measured as bacterial counts (log_{10} CFU/ml) in peritoneal lavage fluid (PLF), blood, spleen and kidneys: 2 h, n = 15; 4 h, n = 9; 6 h, n = 6; 8 h, n = 12; 10 h, n = 9. Slopes (β) from linear regression analysis (0–8 h; blood, spleen and kidneys versus PLF) were not significantly different (p > 0.05), yet elevations (blood, spleen and kidneys versus PLF) were all significantly different (p < 0.0001) (b) Bacterial growth measured as bacterial counts, oriC_{qPCR} and oriC_{mic} in PLF and blood. PLF bacterial count and oriC_{qPCR}: 2 h, n = 15, 4 h, n = 9; 6 h, n = 6; 8 h, n = 12; 10 h, n = 9. Blood bacterial count: 2 h, n = 15, 4 h, n = 9; 6 h, n = 6; 8 h, n = 12; 10 h, n = 9. Blood oriC_{qPCR}: 2 h, n = 12; 4 h, n = 8; 6 h, n = 5; 8 h, n = 12; 10 h, n = 9. oriC_{mic} from 2 and 4 h (blood) are not presented due to insufficient number of microscopically detected cells (Supplementary Table S1). Bland-Altman agreement between oriC_{qPCR} and oriC_{mic} was good (Supplementary Fig. S1). (c) Bacterial cell length (μm) (proxy for cell mass) correlate with oriC/cell, both in PLF and blood. Data represent pooled microscopically detected cells. Inoculum (0 h), n = 500. PLF: 2 h, n = 133; 4 h, n = 55; 6 h, n = 132; 8 h & 10 h, n = 500. Blood: 6 h, n = 164; 8 h, n = 157; 10 h, n = 500; 2 h and 4 h time points are not presented due to insufficient number of cells. Data are presented as mean ± SD. Dotted line represents the limit of detection for bacterial counts. Time (h) represents hours of infection; inoculum is presented as 0 h and extrapolated to PLF data. r: Pearson's correlation coefficient.

demonstrated a shift from a nearly homogenous bacterial population dominated by non-growing, small cells at 0 hours (i.e. the inoculum; oriC/cell = 1), to heterogeneous populations dominated by bacterial cells with ongoing chromosome replications at various levels (oriC/cell ⩾ 2) and various cell sizes at 2 (PLF only), 4 (PLF only), 6 hours of infection (PLF and blood) (Figs 5 and 6). A minor decline in both oriC/cell and cell size was seen at 8 hours, and was even more pronounced at 10 hours of infection. However, unlike during stationary phase in vitro, we did not observe a complete cessation of chromosome replication during the length of infection. At 10 hours of infection (where minimal oriC/cell and cell size were observed) PLF and blood oriC/cell were significantly different from inoculum oriC/cell (p < 0.0001) (Figs 5 and 6). This indicated that fractions of the population were still growing, albeit slowly, at the terminal stage of infection. It has previously been reported, however, that bacteria may cease growth with 2 oriC/cell present. Yet, under such conditions, oriC would go toward 1 (i.e. 2 fully replicated chromosomes), which we do not observe here.

The correlation between oriC/cell and cell size (μm) was very strong, both in PLF (r = 0.95, p < 0.01) and blood (r = 0.98, p < 0.05) bacterial populations (Fig. 4c).

In summary, when net population size was increasing, indicating growth, oriC/cell and oriC were high, and cell sizes were large (0–6 hours of infection) (Fig. 4b,c). After 6 hours of infection growth was gradually beginning to slow down, demonstrated by a slight decrease in both oriC/cell, oriC and cell size (μm). The data indicate the presence of a fixed initiation mass, also during infection in vivo. Unlike during growth in vitro, we did not observe a complete cessation of chromosome replication in vivo (oriC/cell and oriC remained > 1 at all times during
the course of the experiment) (Figs 4b, 5 and 6 and Supplementary Table S1). As demonstrated by the persistent growth heterogeneity (i.e. unbalanced growth), inferring \( \tau \) from \( \text{ori:ter} \) during growth \textit{in vivo} would be deceptive (Figs 5 and 6). However, \( \text{ori:ter} \), as demonstrated, may be used as an independent measure of bacterial growth rate during infection \textit{in vivo}.

**Bacterial growth \textit{in vivo}: mouse intravenous (i.v.) septicaemia model.** To determine whether the bacterial population found in the bloodstream during peritonitis represented cells growing independently of that at the primary site of infection or not, we attempted to induce primary septicaemia directly via i.v. inoculation of \( 1 \times 10^6 \) CFU/ml bacteria (ALO 4783). We were unable to establish an infection, as a complete clearance of bacteria from the bloodstream had occurred prior to the initial sample collection time point; 2 hours post bacterial challenge (Supplementary Fig. S2). No viable bacterial counts were found in blood, PLF or kidneys at any time. However, in the spleen we observed the presence of a stable bacterial population of (mean (SD)) 3.41 (0.03) log\(_{10}\) CFU/ml of all animals at all times (2–8 hours post challenge), likely as a result of bacterial bloodstream clearance.

None of the animals presented any signs of distress during the observation period, in contrary to the mouse peritonitis model, where signs of distress were starting to appear from 2 hours of infection.

**Discussion**

We have applied and validated two well-established methods for detection of bacterial chromosome replication to shed light on fundamental growth dynamics of \textit{E. coli} during infection \textit{in vivo}. \textit{ori:ter} was determined by qPCR and fluorescence microscopy, respectively, overall with good agreement between methods. The microscopy method has the advantage that it allows for direct observation of \textit{in situ} \textit{ori:ter} at a single-cell level, which can
be used to observe the level of replication heterogeneity within a bacterial population. Also, microscopy can be used to determine cell size, which demonstrated good correlation with the number of replication origins (oriC/cell), in agreement with initiation of chromosome replication taking place at a fixed initiation mass under all growth conditions. However, the fluorescence microscopy method requires precedent fluorescent labelling at

![Graphs and images showing relative frequency distributions of cell length and oriC/cell for different time points.]

**Figure 6.** Relative frequency distributions (%) of pooled microscopically detected bacterial cells (ALO 4783) isolated from peritoneal lavage fluid (PLF) in the mouse peritonitis model; cell length (µm) (left panel) and oriC/cell (right panel). Inoculum, n = 500. PLF: 2 h, n = 133; 4 h, n = 55; 6 h, n = 132; 8 h, n = 500; 10 h, n = 500. We emphasize that data representing 4 hours of infection are subject to uncertainty, due to low number of detected cells (n < 100). Time (h) represents hours of infection.
relevant chromosome loci, along with a certain bacterial quantity for cells to be detected, which is a limitation to the method. The qPCR method, by contrast, reports only the mean ori:ter of a population, without regard to any possible heterogeneity. However, this method is less time consuming, uncomplicated and inexpensive, compared to whole genome sequencing. We conclude that both qPCR and fluorescence microscopy are valid methods for measuring in situ bacterial growth during infection, and that they complement each other favourably.

Bacterial growth in a closed batch-culture in vitro was shown to be directly related to nutrient availability, as anticipated. Ori:ter levels were high, with a high level of replication heterogeneity, during the first hours of incubation. Subsequently, as the bacterial count increased, we observed a complete cessation of growth within a homogenous population of predominantly non-replicating cells, due to starvation after prolonged propagation. A similar pattern of growth was observed in vivo during infection in the mouse peritonitis model. However, unlike during growth in vitro, we observed no complete cessation of growth in neither PLF nor blood as the bacterial densities reached their maximum in the terminal stage of infection. At this stage (8 to 10 hours of infection), the stagnant development in bacterial count is deceptive, as it gives the perception of growth cessation. Indeed, a complete cessation of growth might never occur during infection in vivo, where bacterial life-sustaining resources presumably are still available as long as the animal is alive. We show in this model that the apparent stationary phase during infection represents fractions of both slowly growing and non-growing cells, conceivably balanced by cells taken out by the host immune system. Hence, it is evident that one cannot extrapolate findings from in vitro growth studies to explain pharmacodynamics in relation to bacterial growth during infection in vivo, particularly not in regard to the apparent stationary phase. The in vitro effect of most commonly used antibiotics, including β-lactams, fluoroquinolones and aminoglycosides, has been shown to correlate to bacterial growth rate.

Chromosome replication as a readout for bacterial growth rate could form the basis for refined future studies on antibiotic bacterial growth rate as a function of bacterial population, without acceleration or significant deceleration; likely as the net result of constant influx- and kill-rate of bacteria.

The observed inability of the same strain to establish an infection upon direct introduction into the bloodstream (as demonstrated by the i.v. septicemia model), suggest that E. coli found outside the primary site of infection (e.g. in the bloodstream) is not constituted by an independently growing population, but rather mirrors the population at the primary site of infection. This is in consistence with previous in vivo studies of Streptococcus pneumoniae in the same experimental model, describing the phenomenon as ‘spill-over’. Conceivably, bacterial cells grow at a given rate at the primary site of infection (e.g. the peritoneal cavity), followed by a translocation through lymphatic drainage into the systemic venous system via the thoracic duct and onward to relevant tissues, such as the spleen and kidneys where bacterial phagocytic clearance and filtration, respectively, take place. Our findings are in accordance with previous studies indicating that E. coli, unlike bacteria such as Haemophilus influenzae, do not grow independently intravascularly. Our data suggest that bacterial cells surviving in the bloodstream remain within the same state of growth as that of bacterial cells at the primary site of infection, without acceleration or significant deceleration; likely as the net result of constant influx- and kill-rate of bacteria. These findings underscore the importance of source control in septicemia, i.e. locating and ensuring antibiotic availability at the primary site of infection, as this is where active bacterial growth is taking place.

In conclusion, we present data supporting the applicability of both qPCR and fluorescence microscopy as valid methods for determination of bacterial growth rate in vivo. The methods could be extrapolated to other infection models and other pathogenic bacteria. Ultimately, it would be evident to pursue the inexpensive and easily accessible qPCR method in a clinical setting to examine bacterial growth rates in infected body fluids, which could prove helpful in evaluating future antibacterial strategies.

Methods

Bacterial strains and inoculum preparations. Escherichia coli ATCC® 25922, a clinical isolate from the American Type Culture Collection (Manassas (VA), USA) and CLSI and EUCAST control strain for antibiotic susceptibility testing, was used throughout the study. This strain was applied in vitro both as a wild-type and as a genetically modified version expressing fluorescent fusion-proteins at chromosomal sites corresponding to oriC and terC, respectively (ALO 4783). Only ALO 4783 was applied in the in vivo models.

For in vitro experiments, inoculum from frozen stock cultures were grown overnight in Lysogeny Broth (LB) broth, shaking 140 rpm, at 37 °C in ambient air. Bacterial counts were quantified by spotting 10 μl of 10-fold serial dilutions made in sterile physiological saline in duplicate on LB agar plates.

For in vivo experiments, bacteria were grown from frozen stock cultures overnight at 35 °C in ambient air on 5% blood agar plates (SSI Diagnostica, Copenhagen, Denmark), after which inocula were suspended in sterile physiological saline and quantified by measurement of optical density at 546 nm (OD₅₄₆). Porcine mucin (M-2378, Sigma-Aldrich, Munich, Germany) was added as adjuvant to a final concentration of 5% (wt/vol). The mucin stock solution was prepared in physiological saline, sterilised and adjusted to physiological pH before application. The final inoculum (10⁷ CFU/ml) was quantified by spotting 20 μl of serial dilutions in duplicate on selective bromothymol lactose blue agar plates (SSI Diagnostica, Copenhagen, Denmark).

Construction of double-loci fluorescent-labelled strain. For microscopic visualisation of cellular origins and termini, transgenes were inserted into the ATCC 25922 chromosome, by use of the lambda red recombination technique previously described. P1parS::KAN was PCR amplified from plasmid pMS24 and inserted into the chromosome in the oriC region by use of lambda red recombineering plasmid pTP23, using primers 5′-CATCATTAAAGTGCTTGACCTGAATAACGATAACGAAAGCCGTATTAAAGCCGAAGCTTAAACTT-3′ and 5′-GGTAAATTAGCGATAGTCCAGTGAACAGCCCTTCCGGAGGAGAACCTCAATGCATCCCCTTA-3′. Also, pMT1 parS::CHL was PCR amplified from plasmid pGBkD3-parSpMT1 and inserted, by the same method, into the chromosome in the terC region using primers 5′- ATATAAATTCTAATAATTAGATGTATCTTTCCAT

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TTACGGCGTGGAGCTGCTGCTC-3' and 5'-TCGGTGAGATGCTTTACGTCCTCAAGCCCTCCTTCGCTTACTGTCGGGAATTCC-3'.

Furthermore, the pTrc-mCherry-pMTparB-GFP-P1parB cassette was PCR amplified from pJFM4 and inserted into the chromosomal attT7 site at the 3' end of the glmS gene, using the protocol for Tn7 transgene insertion as previously described, resulting in strain ALO 4783. Co-expression of GFP-labelled P1-ParB proteins and mCherry-labelled pMT-ParB proteins in live cells allowed for visualisation of origins as GFP foci (green) and termini as mCherry foci (red), respectively. Correct insertion of all transgenes was confirmed by PCR.

It should be noted that a substantial part of the LacI operator on pRN010, from which the pTrc-mCherry-pMTparB-GFP-P1parB cassette on pJFM4 originated, had unintentionally been lost during plasmid construction. Thus, the use of IPTG (isopropyl β-D-1-thiogalactopyranoside) for induction of the fluorescence fusion proteins proved redundant.

In vitro experiments. Overnight liquid cultures of both wild-type and genetically modified version of ATCC 25922 with a bacterial density of 10^6 CFU/ml were diluted 1:10,000 into fresh media and grown with shaking 140 rpm, at 37°C.

Growth was observed by repeated measurements of optical density at 600nm (OD_{600}). Samples for quantification of bacterial count, qPCR analysis and fluorescence microscopy were withdrawn at time points 2, 4 and 6 hours of incubation in the exponential growth phase study, and at times 6, 8 and 10 hours of incubation for the stationary growth phase study, respectively. All samples were immediately set on ice after withdrawal. Both studies were performed in duplicate (i.e. including both wild-type ATCC 25922 as control of growth, and the genetically modified version, ALO 4783) in three independent experiments. As both growth curves and oritier were the same for both versions of the strain, these results were pooled for statistical analyses. For fluorescence microscopy, only ALO 4783 was applied.

Experimental animal models. Mouse peritonitis model. The mouse peritonitis model has been previously described and studied extensively. Here, outbred female NMRI mice (Bomfalc; NMRI; weight 26–30 g; Taconic, Denmark) were applied throughout the study. The animals were kept in Macrolon type III cages in groups of three and allowed free access to feed and water. Experiments were initiated after an acclimatisation period of 5 days.

Inoculation was performed by intraperitoneal (i.p.) injection of 0.5 ml bacterial suspension containing 10^6 CFU/ml and 5% (wt/vol) mucin. At various time points of infection, the mice were anaesthetized with a subcutaneous (s.c.) injection of pre-mixed Zolazepam/Tiletamin (Zoletil, Virbac, Kolding, Denmark), Xylasin (Xysol Vet., ScanVet, Fredensborg, Denmark) and Butorphanol (Torbugesic Vet inj., Orphion Pharma, Copenhagen, Denmark) prior to blood collection from total cardiocentesis, performed with a 30-gauge needle via subxiphoid access. Blood was stored in EDTA covered micro tubes (Sarstedt, Nümbrecht, Germany). After euthanasia by cervical dislocation, a peritoneal lavage was performed by injecting 2.0 ml of sterile physiological saline i.p. After 1 minute of abdominal massage, the peritoneum was opened and peritoneal lavage fluid (PLF) withdrawn with a pipette. Spleen and both kidneys were surgically removed using sterile procedures and placed in Eppendorf tubes.

All specimens were immediately placed in an insulated 4°C cooling box for transportation and kept on ice at 4°C until application in subsequent tests. Standard bacterial quantification and DNA purification were performed within two hours; fluorescence microscopy the subsequent day. To ensure that bacterial cells in the blood and PLF specimens did not undergo any alterations in any of the growth parameters measured (oritier, oritC/cell or cell size) while kept on ice at a non-permissive growth temperature\(^{45}\) post harvesting, we performed a confirmatory in vitro experiment where chromosome replication analyses (qPCR and fluorescence microscopy) were performed on exponentially growing cells left on ice for up to 24 hours (Supplementary Materials). The results combined confirmed that storage up to 24 hours on ice at non-permissive growth temperatures did not alter these parameters (Supplementary Fig. S3).

The mouse peritonitis model was repeated in a total of 6 independent experiments, where groups of three cohabitant animals were sacrificed at various combinations of time points; 2 (n = 15), 4 (n = 9), 6 (n = 6) 8 (n = 12) or 10 (n = 9) hours of infection.

Data from repeated experiments were pooled for statistical analyses.

Mouse intravenous septicaemia model. To examine the possibility of inducing primary bacteraemia without an established infection outside of the bloodstream, we applied a direct intravenous (i.v.) septicaemia mouse model. Mice, housing, acclimatisation and feeding procedures were identical to that of the mouse peritonitis model. However, here, the mice were inoculated via i.v. injection of 0.2 ml bacterial suspension containing 10^6 CFU/ml, without adjuvant, into the lateral tail vein. Animals were subsequently handled as above mentioned, with the extraction of blood, peritoneal wash fluid, spleen and kidneys, which were all kept at 4°C after harvesting. The animals were sacrificed in groups of three biological replicates at 2 (n = 3), 4 (n = 3) and 8 (n = 3) hours of infection.

Ethics statement. Mice in both experimental models were regularly observed and scored for signs of distress. Humane end points were constituted by signs of irreversible sickness. The mice would be euthanized upon presentation of any of these signs. All animal experiments were approved by the Danish Animal Experimentation Inspectorate (Licence No. 2014-15-0201-00171) and performed according to institutional guidelines.
Quantification of bacterial growth. Samples from in vitro experiments were spotted (10 μl) in duplicate of serial dilutions on LB agar plates, and the bacterial count was recorded as the mean of two plates after overnight incubation at 37 °C in ambient air. The detection limit was 100 CFU/ml.

To the tubes containing spleen or kidneys from the in vivo experiments, sterile physiological saline was added to a total volume of 1 ml, after which they were homogenised for 2 minutes at 30 oscillations/sec using a TissueLyser II (Qiagen, US), before being spotted (20 μl) in serial dilutions in duplicate on bromothymol lactose blue agar plates. The required addition of saline before homogenisation of the tissues entailed a dilution factor of approximately 1:1. Blood and peritoneal wash fluid from in vivo experiments were spotted directly, without further dilution. Colony counts were performed after overnight incubation at 35 °C in ambient air and recorded as the mean of two plates. The detection limit for blood specimens was 50 CFU/ml. The other materials were recorded as CFU/ml of solution.

Quantitative PCR (qPCR). oriC_{ter} was calculated as the population mean level of qPCR amplified oriC to terC, respectively.

Samples from in vitro experiments were prepared by pelleting 1 ml of culture by 5 min centrifugation at 15,000 × g, after which bacterial cells were re-suspended in 100 μl 10 mM Tris pH 7.4 and 900 μl 77% Ethanol and kept at 4 °C until qPCR was performed. Here, cells were spun down and re-suspended in serial dilutions of sterile DNA/RNA free water prior to analysis.

Bacterial DNA from blood and peritoneal lavage fluid from in vivo experiments were purified for qPCR using QIAamp DNA Mini Kit (51304, Qiagen, Hilden, Germany), according to the manufacturer’s instructions. We were unable to purify adequate quantities of bacterial DNA from spleen and kidney tissue for use in this analysis, possibly due to co-purification of murine DNA and/or low bacterial counts in the tissues.

qPCR was performed as previously described, using primers 5′-GGCAACAGCATGGCGATAAC-3′ and 5′-TTTGATATCCGCTGTACGTA-3′ for partial amplification of the highly conserved gidA gene located immediately leftwards of oriC (representing the terC region), and primers 5′-TCAAGTCGACGACGATGAAT-3′ and 5′-TTGAGCTGCGTCTATCGAG-3′ for partial amplification of the dcp gene, located in close proximity to terC, opposite the oriC region (representing the terC region), respectively.28

The analysis was performed using Takara SYBR Premix Ex Taq II (RR820A, Takara Bio, Saint-Germain-en-Laye, France) in a BioRAD CFX96 (95 °C 30 s, 39 × (95 °C 5 s + 58 °C 30 s), 95 °C 15 s, 60 °C 60 s, 95 °C − 15 s, 65 °C − 5 s, 95 °C − ∞), as previously reported.40 The oriC_{ter} was calculated using comparative cycle threshold (Ct) analysis; the 2^{ΔΔCt} method. A fixed sample of the same strain grown into late stationary phase, where the population would be expected to have an oriC_{ter} corresponding to 1, was used for normalisation in every cycling run. Each biological replicate was run as a minimum as three technical replicates in each cycling run, and the mean Ct value of the technical triplicates (or more) was used to calculate the oriC_{ter}. DNA/RNA-free water was used as negative control template in each run. Furthermore, QIAamp PCR purified blood and peritoneal lavage fluid specimens without viable bacterial growth were regarded as negative controls for blood and peritoneal lavage fluid derived specimens. There was a qPCR detection limit corresponding to approximately 10^3 CFU/ml for purified blood specimens. Hence, not all purified blood specimens yielded adequate qPCR results. No detection limit was observed in samples from in vitro experiments.

Fluorescence microscopy. oriC_{mic} was calculated from automated photomicrographic detection of pooled single bacterial cells carrying intracellular fluorescent markers corresponding to oriC and terC, respectively.

For phase contrast and fluorescence microscopy, bacterial cells were spun down for 2 minutes at 6,000 × g, re-suspended in a small volume of AB minimal medium47 and mounted on microscope slides covered with a thin 2% (wt/vol) AB minimal medium agarose pad.

Blood specimens from in vivo studies underwent murine cell lysis prior to pelleting, by the addition of cold, sterile water mixed with the sample at a ratio of approximately 1:1 for 1 minute. Harvested tissues were not observed in the microscope.

A total of 500 live bacterial cells were pooled and analysed from each time point, with the exception of certain specimens, where this was not possible due to the quantity of live cells available for microscopy detected being too low (Supplementary Table S1). The oriC_{mic} was deduced from the mean of oriC foci detected divided by the mean of terC foci detected.

It should be noted that fractions of the bacterial cells were recorded with a number oriC copies = 2^n (n = 1, 2, 3). The risk of underestimation of co-localising oriCs is a known microscopy resolution limitation in photomicrographic foci quantification.48 It is unlikely that the bacterial cells propagating in vitro underwent asynchronous replication initiation4. Accordingly, cells reported with an oriC cell of 3 in all likelihood represent 4 oriC/cell, and cells reported with an oriC/cell > 4 in all likelihood represent 8 oriC/cell (Fig. 3). Since less is known about chromosome replication during infection in vivo, we cannot rule out any possible replication initiation asynchrony in bacterial populations from the in vivo experiments (Figs 5 and 6).

Medial axis cell length (µm) was recorded by automated detection and used as surrogate measure for cell mass. Images were acquired with a Zyla 5.5 s CMOS camera attached to a Nikon Eclipse Ti-E inverted microscope and analysed with NIS-ELEMENTS and Microbief software for automated bacterial cell and fluorescent foci detection and quantification.49

Statistical analyses. Bacterial quantification data were log_{10} transformed prior to analysis. D’Agostino and Pearson omnibus normality test was applied to all data sets. In general, the bacterial count and the qPCR data sets represented a normal distribution. Cell length and numbers of oriC foci detected by microscopy did not all meet the assumptions for normal distribution. Linear regression analysis was used to compare bacterial counts or
oritC between different materials harvested in the in vivo experiments. Additionally, two-way ANOVA followed by Sidak’s multiple comparisons test was applied within the same data sets to test difference between blood and peritoneal oritC at each time point. Pearson’s correlation coefficient was applied to estimate correlation between cell lengths and oritC/cell. The Kolmogorov-Smirnov test was applied to test differences in oritC/cell distributions between bacterial populations in the blood and PLF, respectively, at different time points. Bland-Altman analysis was performed to evaluate agreement between the qPCR and the microscopy method for detection of oritC. A two-tailed p value < 0.05 was considered significant.

GraphPad Prism version 7 (GraphPad Software, CA, USA) was applied for statistical analysis and illustration.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References


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Author Contributions

N.F.-M., A.L.-O., G.C. and M.S.H. conceptualised the study. N.F.-M. and A.L.-O. secured funding. M.S.H. carried out experiments, performed statistical analyses and drafted the manuscript. A.L.-O., N.F.-M. and G.C. provided critical analysis and discussions. All of the authors reviewed, approved and contributed to the final version of the manuscript.

Additional Information

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Supplementary Information

Chromosome replication as a measure of bacterial growth rate during

*Escherichia coli* infection in the mouse peritonitis model

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Supplementary Methods

**In vitro experiment**

To ensure that bacterial cells in the biological specimens (blood and peritoneal lavage fluid (PLF)) harvested in the *in vivo* experiments and subsequently applied in chromosome replication analyses (qPCR and fluorescence microscopy) did not undergo any alterations in growth post harvesting, while kept on ice at 4°C, we performed an additional *in vitro* experiment where chromosome replication analyses were performed on exponentially growing cells after various amounts of time on ice.

An overnight liquid culture of ALO 4783 with a bacterial density of $10^9$ CFU/ml was diluted 1:10,000 into fresh Lysogeny Broth (LB) and grown with shaking 140 rpm, at 37°C. Growth was observed by repeated measurements of optical density at 600 nm ($OD_{600}$). At $OD_{600}$ of approximately 0.4, samples were withdrawn in triplicates and placed on ice at 4°C. To mimic the change in growth environment that the bacterial cells in the peritoneum underwent (sterile isotonic saline suspension; see Methods in article), half of the samples were spun down for 3 minutes at 6000 rpm, after which the pellet was re-suspended in sterile isotonic saline (NaCl), before being placed on ice. The other half was placed on ice without change of media (LB). At $t = 20$ min, 1 h, 2 h and 24 h the triplicate samples withdrawn from ice and analysed by qPCR ($ori:ter_{qPCR}$) and fluorescence microscopy ($oriC$/cell and cell length (µm)), as described in Methods in the article. Reference samples ($t = 0$ h) were analysed immediately, without standing on ice.

Kruskal-Wallis test followed by Uncorrected Dunn’s test was performed on the data sets to test any difference compared to the reference samples. A two-tailed $p$ value < 0.05 was considered significant.
Supplementary Figure S1. Bland-Altman plot of agreement between the two methods for detection of replication ratio (qPCR and fluorescence microscopy). The plot is presented as difference (ori:ter_{mic} - ori:ter_{qPCR}) versus average (ori:ter_{mic} and ori:ter_{qPCR}) for in vitro and in vivo (mouse peritonitis model) data combined. There was a bias (SD) of 0.07 (0.15) and 95% limits of agreement (presented as dotted lines) from -0.37 to 0.23. There was no systemic variation over the range of measurements. Data from 2 (blood) and 4 (blood and peritoneal lavage fluid (PLF)) hours of infection (in vivo) were not included in the analysis due to insufficient number of microscopically detected cells (n < 100, see Supplementary Table S1).
Supplementary Figure S2. Bacterial growth (ALO 4783) at various anatomical sites after attempted induction of septicaemia in the mouse intravenous (i.v.) septicaemia model (in vivo). Each symbol represents the mean bacterial count (log_{10} CFU/ml) per specimen (animal), withdrawn at 2 (n = 3), 4 (n = 3) and 8 (n = 3) hours (h) post inoculation, respectively. Dotted line represents the limit of detection.
Supplementary Figure S3. Bacterial chromosome replication status of bacterial cells (ALO 4783) withdrawn during exponential growth in LB batch culture and left standing on ice at 4°C. (a) ori:ter<sub>qPCR</sub> of triplicate samples left on ice (20 min, 1h, 2h or 24h), with (LB) or without (NaCl) change of media. Data are presented as mean (SD), relative to the reference (0h). n = 3 per time point. There was no significant difference ($P > 0.05$) in absolute ori:ter<sub>qPCR</sub> between any of the samples left on ice and the reference samples. (b) Microscopically detected bacterial cell length (µm) and oriC/cell from triplicate samples left on ice (20 min, 1h, 2h or 24h), with (NaCl) or without (LB) change of media. Data are presented as the mean of a total of 100 pooled microscopically detected cells, relative to the reference (0h). There was no significant difference ($P > 0.05$) in absolute oriC/cell or cell length (µm) between any of the samples left on ice and the reference samples, with the exception of a marginally significant difference ($P < 0.05$) in cell length(µm) between the NaCl samples left on ice for 20 minutes and the reference samples. As the same bacterial cells (NaCl 20 min) do not differ from the controls in neither oriC/cell nor ori:ter<sub>qPCR</sub>, the latter finding is not considered relevant.
Supplementary Table S1 | Overview of fluorescence microscopy, qPCR and bacterial count results from *in vitro* and *in vivo* (mouse peritonitis model) experiments

<table>
<thead>
<tr>
<th>Sample origin and time point (hours of incubation / infection)</th>
<th>Fluorescence microscopy</th>
<th>qPCR</th>
<th>Bacterial counts</th>
</tr>
</thead>
</table>
|                                                               | *oriC* /cell Mean (SD)  | *terC* /cell Mean (SD) | *ori:ter* *mic* Cell length (µm) Mean (SD) | *ori:ter* *qPCR* Mean (SD) | Log₁₀ CFU/ml Mean (SD) | n
|                                                               | Mean (SD)               | Mean (SD) | (µm) | Mean (SD) | n | (SD) | n |
| Inoculum used for *in vitro* experiments                      | 1.23 (0.44)             | 1.11 (0.33) | 1.10 | 2.57 (0.57) | 500 | 1.04 (0.07) | 6 | 9.31 (0.36) | 6 |
| *In vitro*, 2h                                               | 3.05 (1.22)             | 1.31 (0.46) | 2.33 | 4.03 (1.05) | 147 | 2.62 (0.41) | 6 | 5.61 (0.10) | 6 |
| *In vitro*, 4h                                               | 3.48 (1.13)             | 1.14 (0.35) | 3.06 | 4.23 (1.02) | 500 | 3.13 (0.55) | 6 | 7.85 (0.23) | 6 |
| *In vitro*, 6h                                               | 1.42 (0.60)             | 1.01 (0.09) | 1.40 | 3.45 (0.78) | 500 | 1.50 (0.19) | 12 | 9.22 (0.23) | 12 |
| *In vitro*, 8h                                               | 1.17 (0.39)             | 1.02 (0.15) | 1.14 | 2.59 (0.68) | 500 | 1.01 (0.07) | 6 | 9.54 (0.08) | 6 |
| *In vitro*, 10h                                              | 1.14 (0.35)             | 1.03 (0.18) | 1.09 | 2.24 (0.51) | 500 | 1.06 (0.09) | 6 | 9.61 (0.14) | 6 |
| Inoculum used for *in vivo* experiments                       | 1.15 (0.37)             | 1.05 (0.22) | 1.09 | 2.43 (0.57) | 500 | 1.19 (0.09) | 6 | 6.05 (0.09) | 6 |
| *In vivo*, 2h, Peritoneal lavage fluid (PLF)                  | 2.46 (1.31)             | 1.12 (0.33) | 2.20 | 3.96 (1.16) | 133 | 2.50 (0.36) | 15 | 5.88 (0.31) | 15 |
| *In vivo*, 2h, Blood                                         | 3.00 (0.89)             | ND⁹a       | ND⁹a | 4.06 (1.06) | ⁹a | 2.41 (0.45) | 12 | 4.64 (0.90) | 15 |
| *In vivo*, 4h, PLF                                           | 2.16 (0.97)             | 1.16 (0.37) | 1.85 | 3.68 (1.21) | ⁹a | 1.93 (0.28) | 9 | 6.30 (0.66) | 9 |
| *In vivo*, 4h, Blood                                         | 2.82 (1.33)             | 1.72 (0.65) | 1.64 | 3.97 (0.53) | ⁹a | 1.58 (0.32) | 8 | 4.87 (0.77) | 9 |
| *In vivo*, 6h, PLF                                           | 2.15 (1.03)             | 1.25 (0.52) | 1.72 | 3.18 (0.92) | 132 | 2.06 (0.12) | 6 | 6.83 (0.58) | 6 |
| *In vivo*, 6h, Blood                                         | 2.10 (0.79)             | 1.31 (0.49) | 1.61 | 3.09 (0.73) | 164 | 1.76 (0.22) | 5 | 5.32 (1.19) | 6 |
| *In vivo*, 8h, PLF                                           | 1.90 (0.78)             | 1.16 (0.39) | 1.64 | 3.14 (0.77) | 500 | 1.66 (0.30) | 12 | 7.79 (0.81) | 12 |
| *In vivo*, 8h, Blood                                         | 1.99 (0.82)             | 1.27 (0.48) | 1.57 | 3.17 (0.72) | 157 | 1.67 (0.29) | 12 | 6.83 (1.10) | 12 |
| *In vivo*, 10h, PLF                                          | 1.64 (0.76)             | 1.06 (0.28) | 1.53 | 2.76 (0.76) | 500 | 1.48 (0.17) | 9 | 7.54 (1.07) | 9 |
| *In vivo*, 10h, Blood                                        | 1.64 (0.75)             | 1.09 (0.33) | 1.51 | 2.87 (1.04) | 500 | 1.34 (0.24) | 9 | 7.09 (1.88) | 9 |

* a number of pooled microscopically detected bacterial cells
  b number of biological replicates yielding reproducible qPCR results
  c number of biological replicates analysed for qPCR and bacterial count
  d data are subject to uncertainty due to low number (n < 100) of microscopically detected cells
  e ND: not determined, due to insufficient fluorescent (mCherry) signal
7. PAPER II
Comparative Activity of Ceftriaxone, Ciprofloxacin, and Gentamicin as a Function of Bacterial Growth Rate Probed by *Escherichia coli* Chromosome Replication in the Mouse Peritonitis Model

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**ABSTRACT** Commonly used antibiotics exert their effects predominantly on rapidly growing bacterial cells; yet, the growth dynamics taking place during infection in a complex host environment remain largely unknown. Hence, a means to measure in situ bacterial growth rate is essential to predict the outcome of antibacterial treatment. We have recently validated chromosome replication as a readout of in situ bacterial growth rate during *Escherichia coli* infection in the mouse peritonitis model. By the use of two complementary methods (quantitative PCR and fluorescence microscopy) for differential genome origin and terminus copy number quantification, we demonstrated the ability to track bacterial growth rate, both on a population average level and on a single-cell level, from one single biological specimen. Here, we asked whether the in situ growth rate predicts antibiotic treatment effect during infection in the same model. Parallel in vitro growth experiments were conducted as a proof of concept. Our data demonstrate that the activities of the commonly used antibiotics ceftriaxone and gentamicin correlated with pretreatment bacterial growth rate; both drugs performed better during rapid growth than during slow growth. Conversely, ciprofloxacin was less sensitive to bacterial growth rate, both in a homogenous in vitro bacterial population and in a more heterogeneous in vivo bacterial population. The method serves as a platform to test any antibiotic’s dependency on active in situ bacterial growth. Improved insight into this relationship in vivo could ultimately prove helpful in evaluating future antibacterial strategies.

**KEYWORDS** bacterial growth, chromosome replication, *Escherichia coli*, experimental animal model

Studies of antibacterial activity are largely based on laboratory models, where balanced bacterial populations propagate rapidly in well-defined batch cultures with a finite quantity of life-sustaining nutrients. These in vitro models fail to mirror the true growth dynamics of bacterial pathogens taking place during infection in vivo, where growth in a bacterial population appears to be both slower and less homogeneous (1–3). The dependency on active bacterial growth for most antibiotics to exert their effect is acknowledged and has been examined by other investigators, both in vitro and in vivo (4–11). However, these studies were largely based on bacterial count kinetics as a direct measure of bacterial growth rate. This measure could be misleading during infection in vivo, where the net change in the bacterial count is a function not only of growth but also of the elimination of bacterial cells by the host immune system, a factor not taken into account in the bacterial count kinetics method. Moreover, the method fails to report on any growth heterogeneity within the bacterial population. Hence, there is a need for refined means to measure bacterial growth that can extend...
into clinical use. To date, no gold standard method for measuring \textit{in vivo} bacterial growth rate exists. In recent years, it has been demonstrated that it is possible to extract direct measures of \textit{in vivo} bacterial growth rate by analyzing differential genome coverage from whole-genome sequencing data (12, 13). This method circumvents the limitation of a nonquantifiable bacterial elimination factor, as it reports directly on the bacteria’s physiological state. Nonetheless, it reports merely on the mean population growth rate.

Most bacterial chromosomes are circular with a single origin of replication (\textit{oriC}), from where chromosome replication is initiated and carried out bidirectionally toward a single oppositely located terminus (\textit{terC}) during bacterial growth (14, 15). In \textit{Escherichia coli}, it is acknowledged, from decades of \textit{in vitro} studies, that bacterial growth rate is a function of growth conditions and is precisely coordinated with genome replication (16–18). When growth conditions are favorable, overlapping rounds of synchronously initiated bidirectional chromosome replication occur, allowing for the presence of more than two \textit{oriC} (number of \textit{oriC} copies = \(2^n\) [\(n = 2, 3, \) or \(4]\)) in rapidly growing cells (17, 19). In contrast, when growth conditions are disadvantageous, no or merely one round of chromosome replication occurs, allowing for the presence of only one or a maximum of two \textit{oriC} (number of \textit{oriC} copies = \(2^n\) [\(n = 0\) or \(1]\]) in nongrowing or slowly growing cells (19). Hence, the copy number ratio of \textit{oriC} to \textit{terC} (\textit{oriC}:\textit{terC}) reflects the bacterial population growth rate: during rapid growth, larger fractions of cells undergo one or more rounds of chromosome replication (i.e., mean population \textit{oriC} of \(\geq 2\)) and during slow or no growth, only few cells will undergo chromosome replication (i.e., mean population \textit{oriC} of \(\sim 1\)) (20). By the use of two complementary methods for measuring \textit{oriC}:\textit{terC}, quantitative PCR (qPCR) and fluorescence microscopy, we have been able to probe \textit{in situ} growth rates of fluorescently labeled \textit{E. coli} ATCC 25922, both on a population average level (by qPCR) and on a single-cell level (by fluorescence microscopy) during widespread infection in the mouse peritonitis model (3). We demonstrated a correlation between \textit{oriC} and bacterial cell size at all growth rates and the ability of \textit{oriC}:\textit{terC} to predict the development in net bacterial population size. Moreover, in this recent observation of growth dynamics during host infection, we found that growth rates were largely heterogeneous within the bacterial populations propagating both in the peritoneum and in the blood throughout the duration of infection (3). This finding is consistent with previous reports of \textit{Staphylococcus aureus} growth rate heterogeneity in cystic fibrosis sputum, as measured by isotope tracing (1). These observations underscore the need for refined and easily accessible methods to measure \textit{in situ} bacterial growth rate during various types of infection and its causal relationship with the outcome of antibacterial treatment.

Here, we extended the approach of using chromosome replication as a readout of \textit{in vivo} bacterial growth rate in the mouse peritonitis model to explore its potential in predicting antibacterial treatment effect. For comparison, we chose a representative drug from each of three classes of commonly used bactericidal antibiotics with different cellular targets: ceftriaxone (CRO; a \(\beta\)-lactam), ciprofloxacin (CIP; a fluoroquinolone), and gentamicin (GEN; an aminoglycoside) (21). We hypothesized that the drugs tested would perform better when given during rapid than during slow bacterial growth.

RESULTS

We defined the minimal and maximal growth rates of \textit{E. coli} during infection in the mouse peritonitis model and compared the activities of standardized antibacterial dosing regimens given during either rapid or slow bacterial growth, as defined by qPCR-derived \textit{oriC}:\textit{terC} from infected body fluids. As a qualitative control, fluorescence microscopy was applied for the same materials to demonstrate any treatment-induced physiological change in cell morphology or chromosome replication at a single-cell level. As a proof of concept, corresponding \textit{in vitro} treatment experiments using the same model infective organism, the EUCAST and CLSI reference strain \textit{E. coli} ATCC 25922, were carried out.
**Antibacterial activity as a function of growth rate in vitro.** *E. coli* growth experiments in batch cultures were performed to validate the methods applied in the experimental *in vivo* infection model. For these experiments, we used lysogeny broth (LB), a rich medium that supports rapid bacterial growth, allowing the cells to reach stationary phase due to exhaustion of utilizable carbon sources before any significant physiological alterations occurred (22). Here, stationary-phase bacterial cells were diluted in fresh medium and allowed to grow, with repeated sample collections at 4, 6, 8, and 10 h of incubation. All antibiotics were given as a single dose during rapid or slow bacterial growth. Antibiotic concentrations were standardized, defined from previous studies, and corresponded to serum concentrations observed in humans on standard dosing regimens: ceftriaxone (CRO), 30 mg/liter; ciprofloxacin (CIP), 1 mg/liter; gentamicin (GEN), 10 mg/liter (23–28). Sampling was performed after 2 h of antibiotic exposure. There was a difference in pretreatment bacterial count at the time points chosen for rapid and slow bacterial growth treatment induction, respectively. Hence, antibiotic activity was measured as the difference between pre- and post-treatment bacterial count relative to the pretreatment bacterial count (i.e., relative Δlog_{10} CFU/ml) for comparison.

We tested the wild-type bacterial strain (ATCC 25922) in parallel with the genetically modified derivative of the strain with chromosomally incorporated fluorescent oriC and terC labels (ALO 4783) to ensure the absence of growth retardation due to transgene insertion. As no growth differences were observed (Fig. 1a), *in vitro* data from both versions of the strain were pooled for analysis. In regard to the gentamicin treatment regimen, however, only wild-type ATCC 25922 was applied, as the gentamicin MIC was increased by the presence of the nonremovable kanamycin cassette encoding kanamycin phosphotransferase, used as a clonal selection marker, in ALO 4783 (3, 29) (Table 1).

**Antibiotics administered during rapid bacterial growth in vitro.** The bacterial populations propagating in batch cultures reached maximal growth rates at 4 h of incubation (mean and standard deviation [SD] oriC/terC/H11005 3.13 (0.55)) (Fig. 1b). At this stage, it has been demonstrated that *E. coli* population growth in LB is close to balanced and dominated by large cells growing with overlapping rounds of chromosome replication (3). This is exemplified in Fig. 2A, illustrating a large bacterial cell with multiple oriCs. As a consequence of rapid bacterial growth, there was a subsequent increase in population size (Fig. 1a and b). Between 4 and 6 h of incubation, the growth started to slow down, illustrated by a reduction of oriC/terC at 6 h of incubation followed by a minimal increase in net population size (Fig. 1a and b).

When introduced into batch cultures of rapidly growing cells, all antibiotics resulted in significant bacterial count reduction compared to that of controls (CRO and CIP, P < 0.001; GEN, P < 0.01) (Fig. 1c). Correspondingly, single-cell microscopic analysis of pooled live bacterial cells from treatment batch cultures revealed that the majority of the cells isolated after exposure to either ceftriaxone or ciprofloxacin were affected by the treatment when it was introduced during rapid bacterial growth (Fig. 2C and D, respectively). These bacterial populations were represented predominantly by spherical and filamentous cells (ceftriaxone) or elongated cells (ciprofloxacin); all with multiple fluorescent foci, representing direct or indirect chromosome replication disturbance. Unfortunately, we were unable to accurately quantify the population distributions of oriC and terC due to multiple, overlapping fluorescent foci in these treatment groups. The morphological changes in the ceftriaxone-exposed bacterial populations suggest that ceftriaxone exerted its bactericidal effect through cell wall inhibition predominantly by binding to penicillin-binding proteins (PBPs) 2 and 3, in accordance with previous studies (30). We underscore that the microscopy data from bacterial cultures treated with antibiotics during rapid growth (Fig. 2C to E) are subject to uncertainty, given that only few live bacterial cells (n < 500) were isolated due to the efficient bacterial killing (Fig. 1c). Nevertheless, oriC/terC values extracted from the qPCR data complemented the above-described microscopy findings regarding chromosome rep-
High or\textit{ter} ratios were observed where photomicrographs were dominated by large cells with multiple oriCs and low or\textit{ter} ratios were observed where photomicrographs were dominated by small cells with few oriCs in both treatment and control groups (Fig. 1e and 2A to E). Only ciprofloxacin administered during rapid bacterial growth entailed significantly higher or\textit{ter} ratios than those from posttreatment controls ($P < 0.01$) (Fig. 1e). As to ceftriaxone and gentamicin treatments administered during rapid bacterial growth (i.e., at 4 h of incubation). Controls (CTR) received no antibiotic therapy. For comparison of activities between treatment inductions during rapid and slow growth, data in panels c and d are presented as relative bacterial count reductions. (e) Bacterial growth rates (or\textit{ter}) in pretreatment controls (Pre-T, CTR) and posttreatment controls (Post-T, CTR) (i.e., at 4 and 6 h of incubation, respectively) and in treatment batch cultures after 2 h of antibiotic exposure when therapy was induced during rapid bacterial growth. (f) Bacterial growth rates (or\textit{ter}) in Pre-T, CTR, and Post-T controls (i.e., at 8 and 10 h of incubation, respectively) and in treatment batch cultures after 2 h of antibiotic exposure when therapy was induced during slow bacterial growth. Data in panels c to f are presented as medians and interquartile ranges (IQRs). CTR, $n = 6$; CRO, $n = 6$; CIP, $n = 6$; GEN, $n = 3$. * $P < 0.05$; ** $P < 0.01$; ns, $P > 0.05$ by Mann-Whitney U test.
during rapid bacterial growth, both induced decreases in $ori$ toward $\sim 1$, similar to that of the control group (Fig. 1e). For gentamicin-treated bacterial cells, microscopic visualization of $oriC$ and $terC$ was not possible, as only the wild-type ATCC 25922 was utilized. However, the size and morphology of the cells did not appear to differ from those of the respective control population (Fig. 2E and B).

**Antibiotics administered during slow bacterial growth in vitro.** At 8 h of incubation, minimal bacterial growth rates (mean [SD] $ori$, 1.01 [0.07]) were reached due to nutrient starvation (Fig. 1b). At this stage, it has been demonstrated that the bacterial population is dominated by small bacterial cells without ongoing chromosome replication, i.e., a stage with complete or near complete cessation of growth (3). This is exemplified in Fig. 2F, illustrating small bacterial cells with predominantly one $oriC$/cell. Consequently, there was no significant subsequent net population size increase, and all parameters remained largely unchanged by 10 h of incubation (Fig. 1a and b and 2G). When antibiotic regimens identical to those applied during rapid bacterial growth were
introduced into this population of slowly/nongrowing bacterial cells, only ciprofloxacin caused a significant bacterial count reduction ($P < 0.01$), albeit considerably less than when administered during rapid growth, where a near total clearance of cells was observed (Fig. 1c and d). Ceftriaxone and gentamicin treatment effects were absent (Fig. 1d). Correspondingly, photomicrographs were dominated by cells largely unaffected by all three antibiotics, when compared to posttreatment controls (Fig. 2G to J), and no significant change in ori $\text{ter}$ was observed in any treatment group compared to posttreatment controls (Fig. 1f). The ciprofloxacin-induced increase in ori $\text{ter}$ observed during rapid bacterial growth treatment (Fig. 1e and Fig. 2D) was absent when ciprofloxacin was added to a population of cells largely without ongoing chromosome replication (mean population ori $\text{ter} \sim 1$) (Fig. 1f and 2I).

In summary, during controlled bacterial growth in a closed rich medium batch culture, extreme situations of both rapid growth and complete or near complete cessation of growth were provoked. When identical antibiotic treatment regimens were introduced to cultures of bacterial populations growing at either a maximal or minimal growth rate, the dependency of active bacterial growth for all drugs to exert their effect became evident. The significant bacterial load reductions observed when ceftriaxone or gentamicin was added to rapidly growing bacterial populations were lost when identical treatment regimens were induced during slow bacterial growth. Ciprofloxacin, however, was less sensitive to active bacterial growth, as a significant reduction in bacterial load was observed upon administration during both rapid and slow growth.

Antibacterial activity as a function of growth rate in vivo. In the experimental mouse peritonitis model, a total of 54 mice pooled from 4 independent experiments were challenged intraperitoneally with stationary-phase E. coli. All animals developed widespread infection within 2 h postchallenge. Maximal and minimal bacterial growth rates were successfully probed from infected body fluids (peritoneal lavage fluid [PLF] and blood) (3). During propagation in this infection model, bacterial growth was overall slower than that in vitro, yet never came to a complete cessation (i.e., ori $\text{ter}$ remained $>1$ at all times) during the experiment (Fig. 3a). All antibiotics were administered during rapid or slow bacterial growth as a single dose subcutaneously (s.c.) as follows (concentration and optimal pharmacokinetic/pharmacodynamic [PKPD] parameters mentioned in parentheses): ceftriaxone 5 mg (178 mg/kg, time that the drug concentration exceeds the MIC under steady-state pharmacokinetic conditions [$T_{\text{MIC}}$]); ciprofloxacin 0.4 mg (14 mg/kg, area under the concentration-time curve over 24 h in the steady state divided by the MIC [AUC/MIC]); and gentamicin 1 mg (36 mg/kg; AUC/MIC). Antibiotic concentrations were defined from previous studies (23–28) and all were greater than 10 $\times$ MIC (Table 1). Doses were intended to simulate standard human doses of the same antibiotics. However, due to the rapid elimination in mice, the doses were chosen as a compromise between maximum concentration of drug in serum ($C_{\text{max}}$) and either $T_{\text{MIC}}$ (ceftriaxone) or AUC/MIC (ciprofloxacin and gentamicin). Infected biological specimens (PLF, blood, spleen, and kidneys) were harvested after 2 h of antibiotic exposure. For ceftriaxone and ciprofloxacin treatment experiments, ALO 4783 was used as the infective agent. For gentamicin treatment experiments, the ATCC 25922 wild type was applied, for reasons previously explained.

Antibiotics administered during rapid bacterial growth in vivo. The bacterial populations reached maximal in situ growth rates (mean [SD]; ori $\text{ter}$ PLF, 2.54 [0.30]; ori $\text{ter}$ blood, 2.37 [0.46]) at 2 h of infection (Fig. 3a). At this stage of infection, it has been demonstrated that bacterial population growth is heterogeneous (i.e., the population is made up of bacterial cells of various sizes and DNA contents) (3). Figure 4A illustrates a representative large cell with multiple oriCs, isolated from the PLF. As a consequence of average high growth rates, there were subsequent increases in net bacterial population sizes in all biological specimens (Fig. 3a). After 2 h of infection, there was a gradual decrease in ori $\text{ter}$, resulting in overall net population stagnation between 8 and 10 h of infection (Fig. 3a).
When administered during rapid bacterial growth, all antibiotics (CRO, CIP, and GEN) caused significant bacterial count reductions at all anatomical sites examined (PLF, blood, spleen, and kidneys; \( P < 0.01 \)), including a total elimination in blood and kidneys, compared to those in the respective controls (Fig. 3b). The anatomical site-
specific differences in antibiotic activities observed (e.g., between the blood and PLF) cannot be explained by differences in site-specific in situ pretreatment oriC, as these were not significantly different (P > 0.5) (Fig. 3a). Rather, these differences are conceivably attributable to other pharmacodynamic and/or host immune parameters.

We were able to isolate only a few live bacterial cells for fluorescence microscopy from infected body fluids in pre- and posttreatment control groups during rapid bacterial growth (Fig. 4A and B) due to low bacterial counts during the early hours of infection (Fig. 3a). Given the substantial or total clearance of bacteria from the PLF and blood following antibiotic exposure during rapid growth, we were unable to isolate live bacterial cells from these treatment groups. A total of 500 cells were pooled and analyzed per time point from pre- and posttreatment controls during slow growth. For pre- and posttreatment controls during rapid growth, fewer cells were isolated due to low bacterial counts during early hours of infection: n = 142 and n = 66, respectively. For slow growth treatment induction: CRO, n = 170; CIP, n = 35; GEN, n = 228. Due to the limited resolution of fluorescence microscopy for colocalizing oriC foci, some bacterial cells with overlapping chromosome replication origins may appear with too few foci (33). Mean (SD) population medial axis cell lengths were as follows: (A) rapid bacterial growth, pretreatment CTR, 3.96 (1.15) μm; (B) rapid bacterial growth, posttreatment CTR, 3.73 (1.13) μm; (C) slow bacterial growth, pretreatment CTR, 3.19 (0.73) μm; (D) slow bacterial growth, posttreatment CTR, 2.77 (0.86) μm; (E) slow bacterial growth, post-CRO treatment, not determined (due to overrepresentation of spherical cells); (F) slow bacterial growth, post-CIP treatment, 3.25 (0.68) μm; (G) slow bacterial growth, post-GEN treatment, 2.56 (0.76) μm. CTR, controls; CRO, ceftriaxone; CIP, ciprofloxacin; GEN, gentamicin. Scale bar, 2 μm.

**Antibiotics administered during slow bacterial growth in vivo.** Minimal bacterial growth rates (mean [SD]: oriC PLF, 1.53 [0.2]; oriC blood, 1.57 [0.29]) were observed starting from 8 h of infection (Fig. 3a). Marginally lower oriC levels were observed in bacteria isolated from both PLF and blood at 10 h of infection (mean [SD]: PLF, 1.48 [0.17]; oriC blood, 1.34 [0.24]) (Fig. 3a). However, these differences were not statistically significant (P > 0.5), and at this time, the criteria for euthanasia were met for control animals. Hence, 8 h of infection was chosen as the time point for slow bacterial growth treatment induction.

When administered during slow bacterial growth, only ciprofloxacin treatment caused significant bacterial count reductions at all anatomical sites (PLF, blood, spleen, and kidneys; P < 0.01) (Fig. 3c). The activity of gentamicin was overall reduced, and ceftriaxone activity was substantially reduced, at all anatomical sites when antibiotics...
were administered during slow bacterial growth compared to that during rapid bacterial growth (Fig. 3b and c).

Photomicrographs of live bacterial cells exposed to antibiotics during slow bacterial growth indicated that the drugs exerted their effects similar to that in vitro: ceftriaxone inhibited cell wall synthesis predominantly via PBP 2 (demonstrated by the presence of spherical cells) and PBP 3 (demonstrated by the presence of filamentous cells) (30) (Fig. 4E), and ciprofloxacin interrupted natural bacterial growth by inducing cell enlargement, with multiple fluorescent foci, due to interference with ongoing chromosome replication (Fig. 4F) (31). Due to multiple, overlapping fluorescent foci in these treatment groups, we were unable to accurately quantify the population distribution of oriC and terC. For gentamicin-treated bacterial cells, microscopic visualization of oriC and terC was not possible, as the wild-type ATCC 25922 was utilized. However, the size and morphology of the bacterial cells did not appear to differ from those of the respective control population, as observed after antibiotic administration during slow bacterial growth (Fig. 4D and G). We emphasize the uncertainty in these microscopy data (Fig. 4E to G), as only few live bacteria ($n < 500$) were isolated due to antibiotic-induced bacterial killing (Fig. 3c).

The bacterial populations (both in PLF and blood) exposed to antibiotics during late stage of infection (i.e., at 8 h of infection) (Fig. 3a) differed from those observed after prolonged propagation in vitro (i.e., at 8 h of incubation) (Fig. 1b) in that there was no complete cessation of growth in the former. Here, fractions of the population were still undergoing chromosome replication (as expressed by a mean $\text{ori}$: $\text{ter}$ of $\frac{1}{1}$). Hence, the effect of ciprofloxacin on $\text{ori}$: $\text{ter}$ was apparent also upon treatment during slow bacterial growth (Fig. 3e).

In summary, in vivo bacterial growth rates at the time points representing rapid and slow bacterial growth did not differ to the same extent as those during propagation in a rich medium in vitro. Consequently, the difference in antibacterial activity as a function of bacterial growth rate became less explicit. Nevertheless, the overall trends were similar to those observed in vitro: only ciprofloxacin treatment entailed significant bacterial load reduction in all examined body fluids and tissues, both during rapid and slow bacterial growth. Contrary to the in vitro results, however, ceftriaxone and gentamicin both caused a certain bacterial load reduction when administered during slow bacterial growth, albeit less overall than that during rapid bacterial growth. This difference is likely due to the fact that bacterial growth at 8 h of infection was not at a (near) complete arrest, as the $\text{ori}$: $\text{ter}$ remained $\frac{1}{1}$.

DISCUSSION

In this study, we determined the activities of three commonly used bactericidal antibiotics with different antibacterial targets as a function of in situ bacterial growth rate, expressed by differential genome origin and terminus copy number quantification ($\text{ori}$: $\text{ter}$) by qPCR. We demonstrated that the overall activities of both ceftriaxone and gentamicin were substantially lower when administered during slow bacterial growth than when administered during rapid bacterial growth in vivo. Contrarily, ciprofloxacin was less sensitive to bacterial growth rate, as the overall activity remained largely unchanged when going from rapid to slow bacterial growth rate treatment induction in vivo. The findings of ciprofloxacin being less sensitive to bacterial growth rate than $\beta$-lactams and aminoglycosides has been demonstrated by others, however, with the limitation of bacterial growth rate being estimated from net bacterial population kinetics (9, 10). In the parallel in vitro experiments, the difference between rapid and slow bacterial growth rate was more explicit, including complete or near complete cessation of growth as the lower extreme growth rate. When administered during near cessation of bacterial growth, only ciprofloxacin exerted a significant bacterial load reduction, while ceftriaxone and gentamicin lost their effect, in agreement with previous observations where bacterial growth rates were extracted from population kinetics (4, 8). The increase in $\text{ori}$: $\text{ter}$ observed after the administration of ciprofloxacin to populations of rapidly growing cells, both in vivo and in vitro, confirms the drug’s mode
of action. Ciprofloxacin exerts its effect predominantly through DNA gyrase inhibition, which results in the formation of double-strand DNA breaks during chromosome replication, prohibiting the replication forks from reaching the terminus (i.e., the copy number of oriC relative to terC will be high) (31). As anticipated, this effect was lost when ciprofloxacin was introduced into a population without ongoing chromosome replication during slow growth treatment in vitro, as opposed to the slowly growing bacterial populations in vivo, where fractions of cells were still undergoing chromosome replication. As to ceftriaxone and gentamicin, both drugs induced decreases in oriT toward ~1 in rapidly growing bacterial populations, both in vitro and in vivo. Contrary to the respective posttreatment control bacterial populations, however, where similar reductions in oriT were observed, these decreases cannot be explained by natural reduction of bacterial growth rate due to population entrance into stationary phase (i.e., starvation of life-sustaining nutrients due to high population density), as bacterial counts were reduced during antibiotic treatment. Rather, the ceftriaxone- or gentamicin-induced oriT reduction toward ~1 is likely the result of preferential elimination of fractions of rapidly growing bacterial cells (i.e., those with oriT of >1). Hence, besides allowing for measurement of pretreatment in situ bacterial growth rate, oriT may, to some extent, demonstrate the antimicrobial mode of action by analysis of posttreatment oriT. Fluorescence microscopy can complement these findings by direct single-cell visualization, as demonstrated, yet is limited by the absence of live bacterial cells after efficient bacterial elimination.

The growth rate scenarios observed during bacterial propagation in vitro were not representative of the bacterial growth dynamics taking place during infection in a complex host environment. In the latter, a complete or near complete cessation of bacterial growth was not observed as long as the host was alive and bacterial life-sustaining nutrients presumably not exhausted. Hence, for more meaningful prediction of antibiotic activity in vivo, it is important to be able to test this in relation to the in situ bacterial growth rate taking place during host infection rather than extrapolating from in vitro studies. oriT provides predictive value in informing on the likelihood of antibiotic activity, both during E. coli propagation in vitro and during host infection in vivo. There are, however, limitations to be considered in this study. Maximal bacterial growth rates were observed after a few hours of propagation, while minimal growth rates were only observed after prolonged propagation, both in vitro and in vivo. Consequently, the net bacterial population sizes were larger during slow than during rapid bacterial growth. For a meaningful comparison of the effect between identical antibacterial treatment regimens applied, we calculated the relative killing effect in both scenarios. We cannot exclude the possibility of an inoculum effect as a factor contributing to the lower antibiotic activity observed during slow bacterial growth. This is, however, a phenomenon mainly observed in β-lactams and rather unlikely to have occurred at the high antibiotic concentrations that were applied, both in vitro and in vivo (32). Moreover, we were unable to adequately purify bacterial DNA from spleen and kidney tissues. Hence, the difference in spleen and kidney antibiotic treatment effect between the two scenarios in vivo is only assumed to result from different in situ bacterial growth rates in these tissues. Yet, as the temporal development of the net bacterial population size in these tissues followed those in the PLF and blood, we find it likely that bacterial growth rates in these tissues would also be higher in the early hours of infection than after prolonged propagation.

We conclude that chromosome replication as a means to measure bacterial growth rate can predict antibacterial treatment outcome. To some extent, it can also elucidate the antibiotic mode of action, as exemplified by the increase in oriT caused by ciprofloxacin-induced double-strand DNA breaks and the decrease in oriT caused by preferential elimination of rapidly growing bacterial cells by both ceftriaxone and gentamicin. While our findings are in agreement with a previously demonstrated causal relationship between in vivo bacterial growth rate and antibiotic activity, previous studies were limited by the methodology; e.g., the bacterial population kinetics method fails to take into account the host elimination factor, and the tracking of bacterial
growth by isotope trace incorporation is largely inconvenient when it comes to pursuing the method in clinical practice. Tracking bacterial growth rate by differential genome origin and terminus quantification by qPCR has the advantage of being accessible and inexpensive and reports directly on the bacterial physiology, circumventing the limitation of the bacterial count kinetics method. Also, growth rates can be probed from a single biological sample, which is convenient in a clinical setting where repeated sample measurement often is difficult. The method could serve as a platform for testing any antimicrobial's activity as a function of pretreatment bacterial growth rate in experimental infection models and could be pursued in a clinical setting to examine bacterial growth rates in infected biological materials. This could in turn prove helpful in evaluating future antibacterial strategies.

MATERIALS AND METHODS

Bacterial strains. Escherichia coli ATCC 25922, a clinical isolate from the American Type Culture Collection (Manassas, VA, USA) and CLSI and EUCAST control strain for antibiotic susceptibility testing, was used throughout the study. This strain was utilized both as a wild type and as a genetically modified version expressing fluorescent fusion proteins at chromosomal sites corresponding to oriC and terC (ALO 4783) (3).

Antimicrobial agents and susceptibility testing. The antimicrobial agents used in this study were procured as the commercial products registered for parenteral use in Denmark: ciprofloxacin (CIP) as ciprofloxacin 2 mg/ml (Fresenius Kabi, Germany), ceftriaxone (CRO) as ceftriaxone Stragen 1 g (Stragen Nordic, Denmark), and gentamicin (GEN) as hexamycin 40 mg/ml (Sandoz, Denmark). Ceftriaxone was dissolved in sterile physiological saline immediately before use. The MICs were determined by antimicrobial gradient strips (Ettest; bioMérieux, France) according to the manufacturer's instructions using a standard inoculum size; i.e., McFarland standard of 0.5, corresponding to \( \sim 10^8 \) CFU/ml.

In vitro batch culture experiments. For in vitro experiments, bacteria were grown in lysogeny broth (LB) as previously described (3). Antibiotics were added to each batch culture at either maximal bacterial growth rate (i.e., at 4 h of incubation) or at minimal bacterial growth rate (i.e., at 8 h of incubation). Samples for quantification of bacterial count, qPCR analysis, and fluorescence microscopy were withdrawn pretreatment (i.e., at 4 or 8 h of incubation at the maximal or minimal growth rate, respectively) and after 2 h of antibiotic exposure (i.e., at 6 or 10 h of incubation at the maximal or minimal growth rate, respectively). All samples were immediately set on ice after withdrawal. Control cultures without antibiotic treatment exposure were included in every experiment.

MICE PEROITONITIS MODEL (IN VIVO EXPERIMENTS)

Mouse peritonitis model in vivo experiments. The mouse peritonitis model was carried out as previously described, using outbred female NMRI mice (weight 28 ± 2 g; Taconic, Denmark) (3). Animals were kept in cages in groups of three; each cage constituting one experimental unit that would be randomly assigned to treatment (CRO, CIP, or GEN) or no treatment (control [CTR]). Antibiotics were administered as a single bolus injection subcutaneously (s.c.) at either the maximal bacterial growth rate (i.e., at 2 h of infection) or the minimal bacterial growth rate (i.e., at 8 h of infection). Sample collections (peritoneal lavage fluid [PLF], blood, spleen, and kidneys) were performed pretreatment (i.e., at 2 or 8 h of infection at the maximal or minimal growth rate, respectively) in control groups and after 2 h of antibiotic exposure (i.e., at 4 or 10 h of infection at the maximal or minimal growth rate, respectively) in both treatment and control groups. Euthanasia and harvesting of biological specimens were carried out as previously described (3). All biological specimens were immediately placed in an insulated 4°C cooling box for transportation and kept on ice at 4°C until application in subsequent tests. Temporary storage of E. coli cultures on ice has previously been demonstrated not to induce any alteration in in situ bacterial growth parameters (cell size, oriC/cell, or oriR) postharvesting (3).

The mouse peritonitis model was repeated in 4 independent experiments, including a total of 54 animals. Data from repeated experiments were pooled for statistical analyses.

ALO 4783 was utilized in all experiments, except for the gentamicin treatment experiment where the ATCC 25922 wild type was used as the infecting agent due to the altered gentamicin MIC in ALO 4783, as mentioned above.

Ethics statement. All animal experiments were approved by the Danish Animal Experimentation Inspectorate (license no. 2014-15-0201-00171) and performed according to institutional guidelines. The mice were regularly observed and scored for signs of distress. Humane endpoints constituted signs of irreversible sickness; the mice would be euthanized upon presentation of any of these signs.

Quantification of antibacterial activity. Bacterial count measurements from in vitro and in vivo experiments were performed as previously described (3). Antibacterial activity was measured as the difference between bacterial counts pre- and posttherapy (Δlog_{10} CFU/ml). For meaningful comparison...
between identical treatment regimens administered at different growth rates (i.e., different pretreatment bacterial loads), antibacterial activity was reported as \( \text{Slog}_{10} \) CFU/ml relative to the pretreatment bacterial count.

**Quantitative real-time PCR.** orfC was calculated as the population mean level of qPCR amplified orfC relative to that of terC from purified bacterial DNA, as previously described (3).

**Fluorescence microscopy.** Fluorescence microscopy was used to verify the qPCR data, whenever possible, by direct observation of live single cells of ALO 4783 carrying fluorescent markers corresponding to the orfC and terC sites. Fluorescence microscopy analysis was carried out as previously described (3). For gentamicin treatment experiments, only cell size and morphology were analyzed, as the ATCC 25922 wild-type strain was utilized. Live bacterial cells were isolated at each sampling time point (i.e., 4, 6, 8, and 10 h of incubation in the *in vitro* experiments, and 2, 4, 8, and 10 h of infection in the *in vivo* experiments). We have previously shown that PLF and blood bacterial population growth rates in this *in vivo* model do not differ (3). Hence, isolated bacteria from PLF and blood were pooled for analysis. We aimed at isolating 500 bacterial cells at each time point, both with and without antibiotic exposure, but this was not always possible due to substantial or total bacterial clearance in many of the treatment groups, as annotated in Fig. 2 and 4.

**Statistical analyses.** Bacterial count data were log\(_{10}\) transformed prior to analysis. D’Agostino and Pearson omnibus normality tests were applied to all data sets. In general, the control group bacterial counts and qPCR data sets represented normal distributions; those of the treatment groups did not. Statistical significance was evaluated by unpaired \( t \) tests for parametric data and by Mann-Whitney \( U \) tests for nonparametric data. A two-tailed \( P \) value of <0.05 was considered significant. GraphPad Prism version 7 (GraphPad Software, CA, USA) was applied for statistical analyses and illustrations.

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We declare no competing interests.

**REFERENCES**


8. PAPER III

Currently under review (January 2019)
Growth rate of *Escherichia coli* during human urinary tract infection inferred from a single biological sample

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**Abstract**

*Escherichia coli* is the primary cause of urinary tract infection (UTI); one of the most frequent human infections. While much is understood about the virulence factors utilised by uropathogenic *E. coli* (UPEC), less is known about the bacterial growth dynamics taking place during infection. Bacterial growth is considered essential for successful host colonisation and infection, and most antibiotics in clinical use depend on active bacterial growth to exert their effect. However, a means to measure *in situ* bacterial growth rate during infection has been lacking. Due to faithful coordination between chromosome replication and cell growth and division in *E. coli*, chromosome replication provides a quantitative measure of bacterial growth rate. Here, we
explored the potential for inferring in situ bacterial growth rate from a single urine sample with
from patients with E. coli bacteriuria by differential genome quantification performed by
quantitative PCR, and sought to determine the contribution of bacterial growth rate to symptoms
or severity of infection. We measured bacterial growth rates from hospitalised patients with E. coli
bacteriuria for up to four days of infection or colonisation and observed overall active bacterial
growth in the majority of the urine samples; however, with both inter- and intra-patient variation.
We were unable to identify a single host parameter to explain these variations. However,
marginally higher growth rates were observed in patients with UTI than without, and in patients
with invasive infection (bacteraemia) than without, suggesting that growth rate could be a factor
contributing to successful infection in humans.

Introduction

The urinary tract constitutes the most common site of human bacterial infection, and Escherichia
coli is by far the most prevalent causative organism at this site (1, 2). Most urinary tract infections
(UTI) result from ascension of bacteria from the urethra to the bladder, and possibly kidneys (3).
Bacterial growth is considered essential for evasion of the host immune response and successful
establishment and maintenance of infection (4). Also, bacterial growth is critical for most
antibiotics in clinical use to exert their effect (5–12). While much is understood about the
virulence of uropathogenic E. coli (UPEC), less is known about the bacterial growth dynamics
taking place during human infection (13, 14). To date, there exists no gold standard method for
probing bacterial growth rate during host infection. Extracting bacterial growth rates from
bacterial count kinetics is convenient, but not during human infection where frequently repeated
sample measurements are not possible, and the contribution of the host immune system to bacterial elimination is not taken into account. However, in recent years, as complete bacterial genome sequences have become broadly available, it has been possible to probe in-host bacterial growth rates by differential genome coverage analyses; from either whole-genome sequencing or quantitative PCR (qPCR) data (15–19). These methods are based on the principle that growth of *E. coli*, like many other bacteria, is precisely coordinated with the replication of its single circular chromosome (20). In *E. coli*, growth-dependent chromosome replication is initiated from a single defined origin of replication (oriC), from where replication is carried out bidirectionally by two replication forks moving toward the opposite located terminus of replication (terC) once per cell cycle (20, 21). However, given beneficial growth conditions, the bacteria grow with overlapping replication cycles, where chromosome replication is initiated synchronously from $2^n$ ($n = 1, 2, 3$) origins; a phenomenon termed multifork replication (22). This allows for doubling times shorter than the replication time. Hence, the copy number of chromosome oriC relative to terC (ori:ter) is positively correlated with bacterial growth rate, as we have recently demonstrated in the murine peritonitis/septicaemia model (19). As a reference, when measured in *E. coli* ATCC 25922 under controlled growth *in vitro*, an ori:ter of ~3 represented maximum growth rate (i.e., a doubling time of approximately 20 minutes) and an ori:ter of ~1 represented no growth (19).

Here, we extended the approach of inferring *in situ* bacterial growth rate from a single biological sample into exploring its potential for use in human urinary tract infection (UTI). We aimed at testing the contribution of bacterial growth rate to symptoms or severity of infection, and the contribution of pretreatment bacterial growth rate to antibiotic effect. We included patients both with and without (i.e., asymptomatic bacteriuria) symptoms of UTI to test for bacterial growth rate
as a possible predictor for successful infection. Urine samples were collected daily for up to four
days to evaluate the temporal development in bacterial growth rate.

Results

Study population

In this study, a total of 31 hospitalised adult patients with significant quantities of *E. coli*
bacteriuria were included. Two patients were later excluded; one due to retraction of patient
consent and one due to revision of the preliminary microbiological identification result. From the
remaining 29 patients, the initial urine sample originally sent to the Department of Clinical
Microbiology for culture (day 0) was collected (Fig. 1, Table 1). Subsequent follow-up urine
samples on day 1, day 2, and day 3 were provided from 25, 12 and 6 patients, respectively (Fig. 1,
Table 1).

In total, 72 urine samples were therefore available for analysis. Patient characteristics are outlined
in Table 1. In brief, the majority of the patients were geriatric (69% were > 70 years old; median
(range) years: 79 (29 – 99)), female (76%), suffering from competing illnesses, and had a short
duration of symptoms (if any) of urinary tract infection (median (range) days: 1 (1 – 14)). Five
patients had disseminated disease (*E. coli* septicaemia). Only three patients received relevant
antibiotics (prophylactic) before collection of the day 0 urine sample, whereas the majority
received antibiotics after collection of the day 0 urine sample; either for suspected UTI or for other
infection (Table 1, Fig. 1). Almost all patients receiving antibiotic treatment received combination
therapy and/or changing regimens.
Bacterial growth rates in human urine (*in vivo*)

Bacterial growth rates, expressed as copy number quantification of *oriC* relative to *terC* (*ori:ter*) by qPCR, were successfully inferred from the day 0 urine sample from 28 out of the total 29 patients (Fig 1, Table 1). From patient no. 23 the volume of urine provided was too low (< 1 ml) for adequate DNA purification (Table 1). We were able to detect *ori:ter* ratios in most, but not all, follow-up urine samples on day 1 – 3, most likely due to significant reduction or elimination of bacteria in the urine after relevant antibiotic treatment (Table 1). With only a few exceptions, there was overall active bacterial growth (i.e., *ori:ter* > 1), with a median (range) *ori:ter* ratio of 1.6 (1 – 3) in the day 0 urine samples, and a median (range) *ori:ter* ratio of 1.65 (1 – 3.9) in the follow-up urine samples (day 1 – 3). Most follow-up urine samples were taken after antibiotic exposure. Hence, the *ori:ter* ratios observed in these bacterial populations could be affected by the antibiotic given, notably concerning antibiotics targeting chromosome replication directly or indirectly, as previously observed in ciprofloxacin-exposed bacteria during experimental murine infection (5). This phenomenon is exemplified in patient no. 6, where a substantial increase in *ori:ter* following exposure to therapeutic doses of Trimethoprim is likely to be explained by a reduction of the nucleotide pool (23) interfering with ongoing chromosome replication and thus preventing replication forks from reaching the terminus (Fig. 1, Table 1) (20). To what extent the *ori:ter* levels probed from urine samples following exposure to other antibiotics represent true growth rates, or whether these *ori:ter* levels were affected by the antibiotic in question is not known. We were able to track natural development in bacterial growth rates (i.e., in the absence of antibiotics) over time in several patients. Natural growth dynamics during UTI and non-UTI are exemplified in patients no. 12, 21 and 24 and patients no. 8 and 26, respectively (Fig. 1, Table 1).
There was overall active bacterial growth in both of these two groups (i.e., ori:ter > 1 in all patients), however, with day-to-day and patient-to-patient variations. In an attempt to evaluate the contribution of bacterial growth rate to symptoms or severity of infection, we compared ori:ter between patients with and without UTI, respectively, and patients with and without invasive infection (E. coli bacteraemia) respectively. Only the day 0 urine samples collected prior to antibiotic treatment were included in this analysis, as the ori:ter ratios inferred from these indeed represented true bacterial growth rates. There were marginally higher bacterial growth rates both in patients with (median ori:ter = 1.7), compared to patients without (median ori:ter = 1.6), symptoms of UTI, and in patients with (median ori:ter = 1.9), compared to patients without (median ori:ter = 1.6), invasive infection. However, neither of these observations was statistically significant (p > 0.05). There was no significant correlation between number of symptom days and ori:ter, and no significant difference in ori:ter in patients with elevated levels of leucocyturia (i.e., > 15 × 10⁶/L) compared to patients without. Due to the absence of antibiotic monotherapy in the majority of the patients included, our data did not allow for meaningful analysis of the contribution of pretreatment bacterial growth rate to antibiotic effect. In summary, we observed both intra- and inter-patient variation in bacterial growth rate, yet no single clinical parameter to explain this variation.

**Bacterial growth rates during controlled propagation (in vitro)**

To determine whether the inter-patient variations in ori:ter observed could be strain-specific, we randomly selected 5 different patient isolates (from patients no. 1, 6, 10, 16 and 29) to undergo controlled growth with a defined inoculation time *in vitro*. We selected isolates from patients with and without UTI to test whether any difference in growth rate could owe to possible different
pathogenicity of the isolates. *E. coli* ATCC 25922 was grown in parallel as a control. These 5 clinical
isolates were subject to whole-genome sequencing. From these data, we were able to distinguish
the isolates by serotype and estimated genome size. The five clinical isolates were all confirmed as
*E. coli* and all were *fimH* positive, allowing for possible expression of type 1 fimbriae required for
successful colonisation of the urinary tract (24). Isolate no. 1 was identified as serotype O2H1, no.
6 as O6H1 and no. 16 as O8H25. Estimated genome sizes were 5.2, 5.0 and 4.9 Mb, respectively.
The O antigens identified in these strains belong to the most frequent O antigens observed in
UPEC (25, 26), and all three patients were classified as having UTI (Table 1). Isolate no. 10 and 29,
however, originated from patients classified as not having UTI and were identified as serotypes
O166H15 and O8H10, respectively (Table 1). The estimated genome size of both was 4.9 Mb.
In this comparative growth experiment, all isolates were incubated in Lysogeny broth (LB) batch
cultures, from where repeated sample measurements were withdrawn hourly for a total duration
of 10 hours, spanning both exponential and stationary growth phases. To complement the
population mean bacterial growth rates measured by ori:ter, we also included single-cell analyses
by flow cytometry: cell mass and total DNA content per cell. From a combination of these
analyses, we were able to demonstrate that the temporal development in growth and the
association between growth parameters were similar in all strains (Fig. 2). All strains exerted
doubling times (\( T_d \)) of approximately 20 minutes during mid-exponential phase (at approximately
3 hours of incubation). These rapid doubling times correlated with a large cell mass, as well as
both a high total DNA content per cell and a high ori:ter; all parameters in accordance with the
presence of bacterial cells growing with overlapping replication cycles (27). After 3 hours of
incubation growth rates gradually declined, as the bacterial populations approached stationary
phase due to starvation of nutrients after prolonged propagation (28).
growth during stationary phase, starting from approximately 6 hours of incubation, was demonstrated by a constant low cell mass and total DNA content, along with ori:ter at its minimum: ~ 1. The development in growth rate measured on a population average (ori:ter) correlated with the development in cell mass measured by single-cell analysis in all isolates, to the same extent as the development in single-cell total DNA did (Fig. 2).

Hence, no strain-specific differences in growth dynamics were observed during controlled growth in vitro.

Discussion

In this study, we aimed at tracking in situ bacterial growth rates from patients with E. coli bacteriuria to better understand the growth dynamics taking place during bacterial propagation in human infection, and to test the hypothesis that successful UTI requires active bacterial growth. Differential genome quantification measurements (ori:ter) inferred from single urine samples provided snapshots of bacterial growth rates, reporting directly on the bacterial physiology, during the course of infection or colonisation in the human urinary tract. From repeated snapshots we were able to provide a dynamic readout of the development in growth rate. In all but one patient, from whom an insufficient amount of urine was available, we were able to isolate bacterial DNA for successful ori:ter copy number quantification. From controlled in vitro growth of randomly selected patient urine E. coli isolates, we confirmed that ori:ter is indeed a robust measure of bacterial growth rate in various clinical E. coli isolates. After induction of antibiotic therapy, however, the ori:ter ratio may be affected by agents targeting nucleic acid synthesis, as observed with Trimethoprim. This observation is in agreement with a previously reported slow-down of
replication kinetics during multifork replication due to experimental nucleotide pool reduction (29).

There were no strain-specific variations during controlled *in vitro* growth that could explain the variation in bacterial growth rate observed between patients; all isolates demonstrated equal potential for rapid growth in a rich medium. Consequently, the variation in ori:ter ratios observed during human infection rather reflects complex host-pathogen interaction. Maximum ori:ter levels observed during growth in a rich medium exceeded those observed *in vivo*, in consistency with previous comparisons between growth rates of *E. coli* ATCC 25922 *in vitro* and *in vivo* during experimental murine peritonitis/septicaemia (19), as well as between various clinical *E. coli* isolates *in vitro* and *in vivo* during experimental murine UTI (15). In this defined medium with a defined inoculation time, the development in growth dynamics followed a predictable pattern: growth rates were high during early hours of propagation, after which growth rates gradually decreased as the population size increased and entered stationary phase due to nutrient starvation. In human bacteriuria, however, the inoculation time is undefined, and the growth conditions in the urine might be subject to host-specific factors, especially in a patient population dominated by significant comorbidity. Urine has in general been considered a good growth medium for *E. coli*, as it contains a variety of inorganic salts and organic compounds and is regularly replenished by fresh urine production (1). Indeed, in a recent report of bacterial growth rates inferred from 8 urine samples from female out-patients with *E. coli* UTI rapid bacterial growth was observed (15). In the same report, higher bacterial growth rates were observed in isolates classified as UPEC than in isolates classified as asymptomatic bacteriuria isolates during experimental murine UTI, suggesting that UPEC isolates could be better adapted to growth in the urine (15). While we observed marginally higher bacterial growth rates in isolates from patients...
with UTI than in those without, and in isolates associated with bacteraemia than in those not,
suggesting that active bacterial growth could be a factor contributing to successful infection, these
observations were not statistically significant. From the patients where dynamic ori:ter readouts
unaffected by antibiotics were available, we observed an overall increase in bacterial growth rate,
irrespective of whether the patient had UTI or not. This suggests that the day-to-day and patient-
to-patient variations observed could be a result of natural variations in bacterial growth rate in
vivo. The overall active bacterial growth observed could owe to the fact that urine samples were
collected during early stage of infection or colonisation, even if we were unable to correlate the
number of UTI symptom days to bacterial growth rate. However, it should be taken into account
that both the reported number of UTI symptom days in many cases was subject to uncertainty as
the patient suffered from multiple conditions and/or was unable to accurately define the number
of symptom days, and that the association between inoculation and onset of symptoms remains
elusive.

In this study, we also aimed at testing the pretreatment bacterial growth rate as a predictor for
antibiotic effect during human infection. We have recently demonstrated a positive correlation
between pretreatment bacterial growth rate and activity of different antibiotics in clinical use in a
murine model of infection (5). In the present study, antibiotic therapy was managed by the
clinician and guided by combined clinical and paraclinical findings. Most patients included in the
study received combination therapy and/or changing antibiotic regimens. Thus, our data did not
allow for meaningful analysis of the association between bacterial growth rate and the effect of a
specific antibiotic. We have, however, demonstrated the successful translation of a robust means
to measure in situ bacterial growth rate from a single biological sample into clinical practice,
allowing for novel insight into bacterial growth dynamics during human infection. The fact that
bacterial growth rate is a dynamic entity underscores the importance of probing pretreatment
growth rate in human infections, as this could have implications for future antibacterial treatment
strategies. There is potential for pursuit in a larger cohort, preferably of patients with
uncomplicated cystitis receiving antibiotic monotherapy, to evaluate the relationship between
pretreatment bacterial growth rate and antibiotic effect in humans.

Materials and methods

Study population and strain collection

From the 1st of January 2018 to the 30th of May 2018 we identified patients who had significant
quantities ($\geq 10^3$ CFU/ml) of *E. coli* cultured from urine samples sent to The Department of Clinical
Microbiology at Copenhagen University Hospital, Herlev, Denmark. Eligible for enrolment were
adult inpatients at the hospital’s Department of Infectious Diseases, Dept. of Gastroenterology,
Dept. of Geriatrics or Dept. of General Medicine, who had had the urine sample taken no more
than one day prior to *E. coli* identification. Following informed consent, a daily urine sample for up
to another three consecutive days was provided from each patient during hospitalisation. The
study population included patients both with and without symptoms of urinary tract infection
(UTI). All urine samples were kept at 4°C after collection.

Research ethical approvals

Approvals for this study were granted by the Danish Regional Committee on Health Research
Ethics (H-17027763) and the Danish Data Protection Agency.
**Bacterial identification and susceptibility testing**

All urines samples routinely sent to the Department of Clinical Microbiology for culture were handled according to standard laboratory practice. Culture for identification and antimicrobial susceptibility testing was performed on Chrom (Brillance UTI agar; CM949, Oxoid) – Columbia 5% (Difco Columbia Blood agar Base + 5% horse blood; 279240, BD) bi-plates and disk diffusion test, according to the European Committee on Antimicrobial Susceptibility Testing EUCAST standards (30) on Mueller-Hinton agar plates, respectively. Species identification was performed by MALDI-TOF; except for the presentation of typical red colonies on Brilliance UTI agar combined with Cefpodoxim susceptibility, as these isolates would be directly identified as *E. coli*. All follow-up urine samples were tested for regrowth of yellow colonies by plating 50 µl in duplicate on bromothymol lactose blue agar plates incubated overnight at 37° C.

**In vitro growth experiment**

For evaluation of possible strain-specific variation in bacterial growth dynamics, we randomly selected 5 of the included *E. coli* isolates (# 1, 6, 10, 16 and 29) for comparison of growth in Lysogeny Broth (LB) batch cultures. *E. coli* ATCC 25922 was run in parallel as a control. Here, an overnight liquid culture of each strain was diluted 1:10,000 into fresh media and grown with shaking 160 rpm at 37°C. Growth was observed by repeated measurements of optical density at 600 nm (OD$_{600}$). Samples for qPCR analysis and flow cytometry were withdrawn hourly from 2 to 10 hours of incubation. All samples were immediately set on ice and fixed by pelleting 1 ml of culture by 5 minutes centrifugation at 15,000 × g, after which bacterial cells were re-suspended in 100 µl 10 mM Tris pH 7.4 and 900 µl 77% Ethanol, and then kept at 4°C until application in downstream analyses. The experiment was independently repeated three times.
Whole-genome sequencing and data analysis

Bacterial DNA from the same 5 randomly selected isolates were extracted from liquid cultures and sequenced using the Illumina platform, according to manufacturer’s protocol. De novo assembly was done with Unicycler software (31) and contigs greater than 500 bp were used to estimate genome size with QUAST (32). Identification and serotyping were performed using tools from the Center for Genomic Epidemiology (33–36).

Flow cytometry

Flow cytometry was performed on samples withdrawn from in vitro experiments, as previously described (37), using an Apogee A10 instrument. On average 30,000 cells were analysed per sample. Measurements of cell mass and total DNA content per cell were recorded and expressed as relative to the cell mass and total DNA content per cell, respectively, of the same strain during late stationary phase (i.e. the sample collected after 10 hours of propagation).

Real-time quantitative PCR (qPCR)

Bacterial DNA from urine samples, concentrated by pelleting of the total amount of urine available (up to 10 ml), was purified for qPCR using QIAamp DNA Mini Kit (51304, Qiagen), according to the manufacturer’s instructions. Fixed samples from the in vitro experiments were prepared for qPCR by pelleting by centrifugation, followed by re-suspension in serial dilutions of sterile DNA/RNA free water.
qPCR was performed as previously reported (19), using specific primers amplifying genes within or in close proximity to oriC and terC, respectively. Due to minor variation in amplification efficiencies of the two amplicons in some of the clinical isolates, we tested two primer pair combinations in parallel in each bacterial isolate. The primer pair combination yielding the most optimal amplification efficiency of both amplicons was chosen for analysis in the respective bacterial isolate. Primers 5’ –CTGTGAATGATCGGTGATCC– 3’ and 5’ –GTGGATAACTCTGTCAGGAAGCTTG– 3’ (ori primer pair #1) (38) and primers 5’ –CGCAACAGCATGGGCGATAAC– 3’ and 5’ –TTCGATCACCCCTGCGTACA– 3’ (ori primer pair #2) (39) were used for partial amplification of the inter-region between gidA and mioC within the oriC region, and for partial amplification of the highly conserved gidA gene located immediately leftwards of oriC, respectively. Primers 5’ –AACTACGCGGGAAATACCC– 3’ and 5’ –TATCTTCCTGCTCAACGGTC– 3’ (ter primer pair #1) (38) and primers 5’ –TCAACGTGCGAGCGATGAAT– 3’ and 5’ –TTGAGCTGCGCTTCATCGAG– 3’ (ter primer pair #2) (39) were used for partial amplification of ynfD/ynfE within the terC region, and for partial amplification of the dcp gene, located in close proximity to terC, respectively.

Expression of oriC relative to terC (i.e. ori:ter) was calculated using comparative cycle threshold (Ct) analysis adjusted according to the exact amplification efficiency for each amplicon (the Pfaffl method) (40). Amplification efficiencies were calculated for both primer pair combinations by linear regression analysis of Ct-values from serial dilutions of every strain grown into late stationary phase. Amplification efficiencies of 90 – 105 % and $r^2$ of > 0.98 were considered acceptable.

A fixed sample of the relevant strain grown into late stationary phase, where the bacterial population would be expected to have an ori:ter corresponding to 1, was used for normalisation in every cycling run. Each biological replicate was analysed by three technical replicates in each
cycling run, and the mean Ct value of the technical triplicates was used to calculate the ori:ter.

DNA/RNA-free water was used as negative control template in each run. Correct qPCR amplification was verified by gel electrophoresis.

Statistical analyses

Statistical significance in nonparametric data was evaluated by Mann-Whitney U test. Correlation by Pearson’s correlation coefficient. A two-tailed p-value of < 0.05 was considered significant.

GraphPad Prism version 7 (GraphPad Software, USA) was applied for statistical analysis and illustration.

Acknowledgements

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The authors declare no competing interests.

Author contributions

All authors contributed to the study design, interpretation of data, critical analysis and discussion. NFM, ALO and MSH secured funding. MSH and FBH were responsible for inclusion of patients. BS and MSH carried out the laboratory experiments. GC analysed the whole-genome sequencing
data. MSH performed statistical analyses and drafted the manuscript. All authors reviewed, approved and contributed to the final version of the manuscript.

References


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<sup>a</sup>No. of UTI symptom days (if any) before collection of day 0 urine sample. NR: not relevant (no UTI symptoms); NA: not available

<sup>b</sup>Patient defined as having UTI or not by the clinician (based on a combination of symptoms, clinical and paraclinical findings)

<sup>c</sup>Any antibiotic given during observation period (either for UTI or other infection)

<sup>d</sup>Patient recovery within observation period as defined by the clinician (based on a combination of symptoms, clinical and paraclinical findings)

<sup>e</sup>ESBL positive isolate

AMX: amoxicillin; AMC: amoxicillin-clavulanic acid; AMP: ampicillin; CMX: cefuroxime; CLR: clarithromycin; GEN: gentamicin; MTZ: metronidazole; MOX: moxifloxacin; PEN: penicillin; TZP: piperacillin-tazobactam; PMC: pivmecillinam; TMP: trimethoprim
FIG 1. Distributions of ori:ter in the study population. Each bar represents the mean ori of technical triplicates relative to the mean ter of technical triplicates analysed from one urine sample. For every patient, the lower bar represents the day 0 urine sample, and the follow-up urine samples (day 1 up to day 90).
3) are presented in chronological order upwardly directed. Urine samples not yielding adequate copy numbers for quantification (indicated in Table 1) are not presented. Arrow indicates that antibiotic treatment (any relevant) had been given at the time of urine sampling (Antibiotics+); no arrow that no relevant antibiotic treatment had been given at the time or urine sampling (Antibiotics-). Closed circle indicates that the patient was classified as having UTI (UTI+); open circle that the patient was classified as not having UTI (UTI-). Dotted line represents the minimum possible ori:ter level.
FIG. 2. Controlled growth of selected *E. coli* urine isolates in Lysogeny Broth (LB) batch cultures *in vitro*. *E. coli* ATCC 25922 was used as control. Bacterial growth measured as optical density (OD$_{600}$) (black triangles), ori:ter (green triangles), cell mass (blue circles) and total DNA content per cell (red squares). The latter two measurements were made by flow cytometry and are presented as relative to those of the same isolate.
during late stationary phase (i.e. the samples collected at 10 hours of incubation). An average of 30 000 bacterial cells were analysed by flow cytometry per sample. Data are presented as mean±SD of three independently repeated experiments. Time (h) represents hours of incubation. Doubling time (T_d) is inferred from OD_600 measurements during mid-exponential growth. Pearson’s correlation between cell mass and total DNA content per cell is shown as r(1), and correlation between cell mass and ori:ter as r(2), respectively. Measurements from 10 hours of incubation were excluded from correlation analyses, due to the fact that these measurements were used as denominator for the flow cytometry data (i.e. relative cell mass and relative total DNA content). ***, p ≤ 0.001; ****, p ≤ 0.0001.
9. DISCUSSION

Many of the advancements within the field of microbiology over the past decades have come from isolating bacteria from the wild and studying their nature under controlled growth in liquid media or on agar plates in the laboratory. The pharmaceutical industry still operates with this *in vitro* approach to mimic the behaviour of bacteria within the host in the development of new antibiotics (143, 144). However, growth conditions within the host are much more complex than those encountered in the laboratory. The behaviour of a pure culture during growth in artificial media does not reflect well the behaviour of bacterial populations growing in the body during infection. For instance, up to as much as 80% of human microbial infections are estimated to be constituted by biofilm infections (145). Bacterial cells within biofilm communities are physiologically different from those within planktonic cultures, often including a mixture of growing cells and cells growing slowly or not at all (145). To truly understand the growth dynamics of pathogens at different sites and how well they respond to antimicrobials during human infection, it is important to study bacterial growth in its natural context; that is, in the body.

9.1 Means to measure bacterial growth rate *in vivo*

As outlined in the introductory paragraph, many investigators in the past have dealt with the *in vitro-in vivo* translation of different approaches to probe the growth rate of different bacteria in different hosts (including mice, rats and humans) and different anatomical sites, with variable success. In general, most of these methods were either based on the dilution of an experimentally introduced marker, which limits their use to experimental models, or required post-sampling processing that could affect the physiology of the bacteria. What we have learned from these reports is that, in general, bacterial growth in the host is often slower than what is observed under optimal growth conditions *in vitro*. However, there are a few exceptions. For instance, quantitative determination of growth rate by the use of temperature sensitive mutants of *P. aeruginosa* in mouse lungs and of *P. aeruginosa* or *E. coli* intraperitoneally in mice revealed maximum average doubling times *in vivo* (calculated from
extrapolation from in vitro data) close to those observed under optimal growth conditions in vitro; 32 min, 20 min and 33 min, respectively (34, 35). Also, the growth rate of E. coli in 8 women with acute cystitis was calculated (by extrapolation from in vitro data using the PTR method) to correspond to an average doubling time of 22 min; similar to the maximum doubling time of E. coli observed in vitro (52). At the other extreme, the doubling time of M. leprae during chronic infection in mouse foot pads was estimated to average 25 days (27).

What remains clear is that within-host bacterial growth rates vary greatly and depend on the given pathogen and its growth conditions (including e.g. nutrient access, competition from other microorganisms and elimination by host immune system or other clearance factors). A direct measurement of in vivo bacterial growth rate can be technically challenging, in large part because of the complex environment, often involving other microbes, the presence of host immune cells and antimicrobial drugs.

9.1.1 Chromosome replication as a measure of bacterial growth rate in vivo

One of the most interesting methods for extracting in situ bacterial growth rate is the PTR method, where growth rate is inferred from differential genome coverage from whole-genome sequencing data, as recently reported by Korem et al. (49). It is a marker-independent method that requires no post-sampling intervention and allows for the measurement of several species simultaneously. There are, however, limitations to this methodology. A PTR readout requires sequencing read mapping to a reference genome, which makes it sensitive to potential assembly errors in the reference database (51, 146). It also requires adequate read depth, which is not always possible in clinical specimens (52). Moreover, it merely reports on growth rate summed across the bacterial population. As we (147), and others (39, 41), have demonstrated from single-cell analyses, growth rate appears to be heterogeneous in bacterial populations propagating within a host during infection.

We have applied a similar approach, based on the classic understanding that E. coli chromosome replication is initiated synchronously from one or more well-defined origins of
replication and that chromosome replication is precisely coordinated with growth, to probe bacterial growth rate during host infection.

In paper I and paper II we applied two different techniques to quantify the copy number of origin of replication relative to the copy number of terminus of replication (ori:ter) during E. coli infection in the mouse peritonitis model; namely qPCR and fluorescence microscopy. Photomicrographic differential visualisation of the oriC and terC region was made possible by chromosomal expression of fluorescently labelled fusion proteins GFP-P1-ParB (green fluorescence) and mCherry-pMT1-ParB (red fluorescence) (148–153). These fusion proteins bind to their respective P1parS and pMT1parS sequence introduced into the oriC and terC region, respectively, by homologous recombineering (154). Hence, fluorescent foci representing the oriC and terC, respectively, can be recognised on a single-cell level. The method has an advantage over other labelling methods, such as fluorescent in situ hybridization, that is works in living cells (155). Also, the cell size and morphology can be assessed. However, the risk of underestimating the total number of oriCs per cell in rapidly growing cells due to co-localising oriCs constitutes a limitation (156, 157). This had, however, little impact on our data, as there was a good correlation between microscopically detected average oriC/cell and average cell size. By combining fluorescence microscopy with qPCR amplification of oriC relative to terC from total bacterial DNA extracted from the bacterial population, we were able to characterise bacterial growth dynamics both on a population average and on a single-cell level in vivo (158, 159). There was good agreement between the methods (ori:terqPCR and ori:termic), and the approach was validated by parallel in vitro growth experiments demonstrating good correlation between all growth parameters.

As reported in paper I, we observed in the mouse peritonitis model: (i) a constancy in initiation mass across growth rates, (ii) parallel ori:ter ratios and bacterial cell sizes in bacteria derived from the primary site of infection to those spread to the blood, despite a significantly lower bacterial load, suggestive of a passive “spill-over” of bacteria from the primary site of infection to the bloodstream (160), (iii) lower maximum ori:ter in vivo than in vitro, accounting for overall lower growth rates in vivo; (v) no absolute cessation of growth during the terminal stage of
infection, where bacterial densities reached their maximum \((\text{ori:ter remained } > 1 \text{ at all times})\), and (iv) growth rate heterogeneity. The fact that growth did not cease and that growth rate heterogeneity prevailed, reinforces the concept of \textit{in vivo} bacterial growth being distinctive from controlled growth \textit{in vitro}, where growth rates where more homogenous and a complete cessation of growth was observed when bacterial densities reached their maximum. The observed growth rate heterogeneity could be of importance for the outcome of antibiotic treatment, given that most antibiotics rely on active growth to exert their effect. Thus, these findings underscore the importance of measuring antibiotic effect as a function of bacterial growth rate \textit{in vivo}.

9.2 Antibiotic effect as a function of bacterial growth rate

The discovery and production of the first antibiotics in the early 20\textsuperscript{th} Century initiated a new era in the history of medicine (161–166). Over the past decades, however, there has been a rapid rise in antimicrobial resistance (167–171). To address this emerging antibiotic crisis, there is a need for developing treatment strategies that not only involves the development of new antibiotics, but also includes more optimal use of existing ones (9, 172). Thus, a better understanding of \textit{in situ} bacterial physiology and its correlation to treatment effect is essential.

Antibacterial treatment in clinical infections is chosen on the basis of \textit{in vitro} susceptibility testing (173). Yet, treatment might not be effective, especially in chronic infections and immunocompromised patients, despite \textit{in vitro} susceptibility towards the antibiotics used (174, 175). Efficient treatment of many infections is undermined by a lack of understanding of the physiological state of the pathogen at the site of infection (41). While it is commonly accepted that rapidly growing bacterial cells are more susceptible to antibacterial killing and inhibition than slowly growing cells, the bacterial physiology is not being examined upon diagnosis of infection (176). In paper II, we have applied three bactericidal antibiotics with different cellular targets (a \(\beta\)-lactam [cell wall synthesis inhibitor]: ceftriaxone, a fluoroquinolone [DNA synthesis inhibitor]: ciprofloxacin, and an aminoglycoside [protein synthesis inhibitor]: gentamicin) to test the differential genome quantification method as a platform to predict antibiotic activity as a function of pretreatment bacterial growth rate \textit{in vivo}. Ceftriaxone, ciprofloxacin and
gentamicin are all examples of the WHO-defined Critically Important Antimicrobials for Human Medicine, meaning that they belong to an antimicrobial class that is the sole, or one of limited available therapies, to treat serious bacterial infections in humans (177). For comparison, antibiotic activity was tested in parallel in both rapidly and slowly growing bacterial populations \textit{in vitro}. Cultures were made stationary from nutrient starvation after prolonged propagation. In the \textit{in vitro} experiment, the difference between rapid and slow growth rate was more explicit than \textit{in vivo}, including complete or near complete cessation of growth \((\text{ori:ter} \sim 1)\) as the lower extreme and \(\text{ori:ter} > 3\) as the upper extreme. Consequently, the difference in antibacterial activity as a function of bacterial growth rate was less explicit \textit{in vivo}. Nevertheless, the overall trends were similar to those observed \textit{in vitro}. The activity of ceftriaxone and gentamicin was overall reduced when average pretreatment bacterial growth rate was low \((\text{ori:ter} \sim 1.5)\), compared to when average pretreatment bacterial growth rate was high \((\text{ori:ter} \sim 2.5)\).

Ciprofloxacin, however, induced significant bacterial load reduction, both during rapid and slow bacterial growth \textit{in vivo}. Our findings extend those of others, where the growth rate of the same bacteria, \textit{E. coli} ATCC 25922, was controlled by carbon-limitation of the growth media \textit{in vitro} (4). Here, the bactericidal effect of different \(\beta\)-lactams, two fluoroquinolones and an aminoglycoside was tested against a non-growing, slowly growing and rapidly growing bacterial population, respectively. In agreement with our observations, both \textit{in vitro} and \textit{in vivo}, only the fluoroquinolones (ciprofloxacin and ofloxacin) exhibited bactericidal activity against a non-growing population, whereas most drugs exhibited bactericidal activity against a growing population; in general with a higher kill rate at optimal growth than at suboptimal growth (4). Also, in a study of an experimental \textit{E. coli} ATCC 25922 biofilm, where cells are considered as non-growing, ciprofloxacin and the carbapenem imipenem were the only active antibiotics, whereas different cephalosporins were inactive against non-growing cells (178). It is established that some antibiotics, such as the older \(\beta\)-lactams, have an absolute requirement for cell growth in order to kill and that the rate of killing is proportional to the growth rate (3, 7, 9, 179, 180). While some of the more advanced \(\beta\)-lactams, fluoroquinolones and aminoglycosides can kill non-growing cells, the effect is observed to be overall better at high growth rates (179). The reason why some antibiotics, such as ciprofloxacin, are less sensitive to bacterial growth rate remains unclear and warrants future investigation. Our measurements of posttreatment
bacterial populations, after treatment induction during rapid bacterial growth, suggest that ceftriaxone and gentamicin have a preference for eliminating actively growing cells \( (ori:ter > 1) \). Whether this is the case for ciprofloxacin is less clear, since the double-strand DNA breaks induced by the drug increases \( ori:ter \) in posttreatment populations, as a result of ongoing replication forks being prevented from reaching the terminus (181).

9.3 Animal models and human disease

Animal models have been employed to mimic human diseases for more than a century. Today, rodents account for the vast majority of all laboratory animals; an estimated ninety percent of research animals are mice (182). They offer advantages by being inexpensive, small and easy to handle and have a short reproductive cycle (24). Moreover, laboratory mice used in research today are very well-characterised: their entire genome has been sequenced (184), they are genetically close to humans and they exist in lines that are both outbred and inbred (i.e., genetically identical) (182). Intraperitoneal inoculation of mice with different microorganisms to produce peritonitis or sepsis has been used for decades to study a variety of properties, such as pathogenesis, virulence, course of infection, immunology and other host-response factors (183, 185). Examining bacteria during propagation in a host is important for illustrating the natural course of an infection, as well as for studying the \textit{in vivo} effect of antibiotics.

Experimental animal models allow for standardisation of infection and treatment regimens. The mouse peritonitis model has been the model of choice for screening of the effect of antibacterial agents \textit{in vivo}, due to its reproducibility and ease of use (160, 185–195). We chose this model for our \textit{in vivo} experiments (\textit{paper I} and \textit{paper II}), as it is a robust model and allows for the study of experimentally defined (in this case, fluorescently labelled) bacteria. An outbred strain was used to ensure a heterogeneous population, which mirrors variation in the human population and ensures the relevance of a possible antibacterial effect (183). Mice reflect human biology well (196). However, that does not necessarily mean that what is true in mice can be directly extrapolated to humans. When bridging mice to men, one should take into consideration that differences in murine and human immunology could have an impact on bacterial physiology (196). Ideally, observations in animals should, whenever possible, be validated in humans.
In paper III we explored the potential for extending the method of inferring in situ bacterial growth rate from chromosome replication status into clinical use. In this study, we included hospitalised patients with *E. coli* bacteriuria (both with and without symptoms of UTI) and collected urine samples for differential genome quantification by qPCR. We chose to include patients with *E. coli* present in the urinary tract, since (i) bacteria are easily extracted from urine, (ii) the specimen is relatively easy to obtain in sufficient quantities and requires little intervention, (iii) repeated samples can easily be collected to follow the growth dynamics during the course of infection and, most importantly, (iv) UTI is one of the most prevalent bacterial infections in humans, contributing to a major health and economic burden. Improved insight into the growth dynamics of *E. coli* during propagation in the urinary tract could possibly aid future treatment strategies. Here, we used *ori:ter* derived by qPCR as an independent measure of bacterial growth rate, as fluorescence microscopy requires genetically modified bacteria and thus limits its use to experimental infection models. A disadvantage to using qPCR alone is that it demonstrates the population average only. However, the technique is easily accessible, as well as less expensive and less time-consuming than whole-genome sequencing, which makes it better adapted to clinical use. To demonstrate its validity, we conducted parallel *in vitro* growth assays of 5 randomly selected clinical isolates. Here, we used a combination of qPCR and flow cytometry to compare cell size, total DNA and *ori:ter* during both exponential and stationary growth phases. Flow cytometry is a technique commonly used to study bacterial cell cycle parameters (26–31). The system determines cell components that are fluorescently labelled (e.g. DNA) by excitation of fixed cells suspended in water, as well as the cell size, which is directly proportional to the light scatter. We found good correlation between all measured growth parameters (i.e., cell size, total DNA per cell and *ori:ter*) at all growth rates, meaning that *ori:ter* correlates with growth rate in these clinical isolates. From repeated urine sample measurements over up to four consecutive days, we were able to provide a dynamic readout of bacterial growth rates during the course of infection. We observed active bacterial growth in most of the urine samples, however, with day-to-day variations that could owe to a natural development in growth kinetics as the infection progresses. We included patients both with and without UTI to test the contribution of bacterial growth rate to
symptoms and severity of infection. There were marginally higher growth rates in patients with UTI (compared to asymptomatic bacteriuria), and in patients with invasive infection (compared to uncomplicated UTI), indicating that bacterial growth rate could be a factor contributing to bacterial pathogenicity, as suggested by others (12, 15, 49). These findings were, however, not statistically significant and warrant future investigation.

10. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We have demonstrated that low bacterial growth rates in vivo might explain reduced antibiotic effect, despite in vitro susceptibility. As demonstrated, infections can, once established, become indolent and contain non-growing or slowly growing bacteria. In the mouse peritonitis model, growth rates decreased after prolonged infection, as the bacterial loads reached their maximum. Other examples of slow bacterial growth include e.g. abscesses and implant biofilms (4, 145). In these cases, antibiotics with bactericidal activity toward non- or slowly-growing bacteria may have an advantage (4). Thus, mapping the in vivo activity of antibiotics as a function of bacterial growth rate is of importance. Differential genome quantification, inferred from a single biological sample by easily accessible and inexpensive qPCR technique and complemented by fluorescence microscopy for single-cell visualisation, has proven to represent a robust readout of in situ bacterial growth rate. Further animal experiments including other clinically relevant antibiotics (also bacteriostatic) are warranted. Moreover, a model of chronic infection, such as a mouse abscess model, could be included to verify whether bacterial growth during infection may come to a complete arrest or not.

We have been able to translate the approach of inferring bacterial growth rate from qPCR-derived ori:ter into clinical use with success. However, due to the relatively small size of the patient cohort included in the clinical study, as well as the variation in treatment regimens, we were unable to evaluate the contribution of bacterial growth rate to antibiotic treatment effect. It would be interesting to expand the study to include a larger cohort of non-hospitalised
patients with UTI, receiving a single antibiotic, to study the effect of a given antibiotic as a function of pretreatment bacterial growth rate in human infection. Although we were unable to compare the contribution of pretreatment bacterial growth rate to the effect of a given antibiotic, being able to detect \textit{in situ} bacterial growth rate from a single biological specimen at the time of diagnosis provides valuable information on \textit{in situ} bacterial physiology and could have future implications for antibacterial treatment strategies.
11. BIBLIOGRAPHY


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