Intergenic evolution during host adaptation increases expression of the metallophore pseudopaline in *Pseudomonas aeruginosa*

Regulating intracellular levels of biological metal ions is essential for all bacterial species, as they are needed for virulence and a range of metabolic processes. Zinc is the second most abundant metal ion in *Pseudomonas aeruginosa*, but little is known about its regulation. Recent studies have identified a novel operon, *zrmABCD* (also called *cntOLMI*), encoding a metallophore system (pseudopaline) involved in zinc acquisition. Expression of this operon has been implicated in human infections and is regulated by the transcriptional regulator Zur (Zn$^{2+}$ uptake regulator). In this study, we show that the intergenic promoter region in front of *zrmABCD* is a target for recurrent adaptive mutations during chronic infection of cystic fibrosis (CF) patients. We characterize the inter- and intraclonal sequence polymorphisms found in the promoter region of the metallophore system and find that most alterations increase promoter activity. One of the evolved promoters displays a more than 10-fold increase compared to the ancestral strain due to the combined effect of an altered binding site of Zur and changes to the RpoD-binding motif. This specific evolved promoter responds differently to changes in metal ion concentrations in chelated medium. We have previously shown that *P. aeruginosa* evolves toward iron acquisition from haemoglobin during long-term CF infections. We hereby provide the second example of adaptive mutations targeting intergenic regions that affect metal ion uptake systems during CF infections, and the first involving zinc uptake. Our results suggest that the scarcity of metal ions (including iron and zinc) is an important evolutionary driver in CF host adaptation.
Interspecies interactions between bacterial pathogens and the commensal microbiota can influence disease outcome. In the nasal cavities, Staphylococcus epidermidis has been shown to be a determining factor for Staphylococcus aureus colonization and biofilm formation. However, the interaction between S. epidermidis and S. aureus has mainly been described by phenotypic analysis, and little is known about how this interaction modulates gene expression.

This study aimed to determine the interactome of nasal S. aureus and S. epidermidis isolates to understand the molecular effect of interaction. After whole-genome sequencing of two nasal staphylococcal isolates, an agar-based RNA sequencing setup was utilized to identify interaction-induced transcriptional alterations in surface-associated populations. Our results revealed differential expression of several virulence genes in both species. We also identified putative non-coding RNAs (ncRNAs) and, interestingly, detected a putative ncRNA transcribed antisense to esp, the serine protease of S. epidermidis, that has previously been shown to inhibit nasal colonization of S. aureus. In our study, the gene encoding Esp and the antisense ncRNA are both downregulated during interaction with S. aureus. Our findings contribute to a better understanding of pathogen physiology in the context of interactions with the commensal microbiota, and may provide targets for future therapeutics.

Transcriptomic profiling of interacting nasal staphylococci species reveals global changes in gene and non-coding RNA expression

Interspecies interactions between bacterial pathogens and the commensal microbiota can influence disease outcome. In the nasal cavities, Staphylococcus epidermidis has been shown to be a determining factor for Staphylococcus aureus colonization and biofilm formation. However, the interaction between S. epidermidis and S. aureus has mainly been described by phenotypic analysis, and little is known about how this interaction modulates gene expression.

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General information

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Application of RNA-seq and Bioimaging Methods to Study Microbe-Microbe Interactions and Their Effects on Biofilm Formation and Gene Expression

Complex interactions between pathogenic bacteria, the microbiota, and the host can modify pathogen physiology and behavior. We describe two different experimental approaches to study microbe-microbe interactions in in vitro systems containing surface-associated microbial populations. One method is the application of RNA sequencing (RNA-seq) to determine the transcriptional changes in pathogenic bacteria in response to microbial interspecies interactions. The other method combines flow cell devices for bacterial cultivation and growth with high-resolution bioimaging to analyze the microscale structural organization of interacting microbial populations within mixed-species biofilms.

Elucidating the Molecular Factors Implicated in the Persistence and Evolution of Transferable Antibiotic Resistance

Being the most diverse and abundant domain of life, bacteria exemplify the remarkable ability of evolution to fit organisms into almost any imaginable niche on the planet. Although the capacity of bacteria to diversify and adapt is fundamental to natural ecosystems and modern biotechnology, the same adaptive mechanisms constantly threaten human health. Less than a century ago, infectious disease was among the most common causes of mortality, but luckily this situation was drastically improved with the introduction of vaccination and effective antimicrobial drugs. Unfortunately, this situation is changing with the rapid emergence of multidrug resistant bacteria that do not respond to our current treatments. This process is to a large extent driven by gene exchange that allows bacteria to rapidly acquire ready-made adaptive features. The aim of this thesis has been to understand the adaptive mechanisms governing the dynamics of bacterial gene-sharing. Specifically, the focus has been on antibiotic resistance genes and their genetic vectors due to the profound implications of these genetic elements in human health. To observe the extend and impact of gene transfer events in a highly relevant natural environment, we looked into the genomes of Escherichia coli longitudinally sampled from the infant gut over the first year of life. Sequence analysis revealed a high degree of genomic plasticity, with frequent gene
acquisition and loss events. While the acquisition of new genetic material is often deleterious, we show that plasmids encoding resistance and virulence factors may indeed be stably maintained in the gut despite imposing a measurable fitness cost to their bacterial hosts in vitro. In two studies investigating the stability of genetic elements, we zoom in on the molecular mechanisms enabling conflict resolution between incoming genetic elements and naive recipient genomes. In both studies, the burden of initially costly genetic elements is ameliorated via adaptive evolution over time. In the case of a large multi-drug resistance plasmid, adaptation happens through IS26 mediated deletions of costly genes that (collaterally) sacrifice the transfer proficiency of the plasmid. For the industrially relevant mevalonate production pathway, we observe similar population-level loss dynamics. Using ultra-deep sequencing we show that the cost-attenuated pathway variants are interrupted by different IS-element insertions that enrich over time due to the fitness benefit of production loss. For both studies, the compensatory activity depends on the host background, and we suggest measures that can harness evolutionary increase genetic stability of the costly production pathway. The final study of this thesis investigates the phenotypic effects of expressing 200 antibiotic resistance genes in E. coli. As the currency of evolution, genes are subject to selection at different levels that may promote or limit their success when transferred to a new host. Through sequence analysis and experimental interrogations, we suggest that functional constrains, rather than sequence composition, is the main challenge that acquired genes encounter when transferred across phylogeny. The work conducted in this thesis provides novel insight into the persistence and evolution of highly relevant genetic elements in vitro, in vivo and in situ. The conclusions shed light on fundamental evolutionary questions of genome dynamics and bacterial adaptation, which may ultimately improve our ability to predict and prevent the spread of antibiotic resistance and guide the engineering of robust biological systems.

General information
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Genomic epidemiology of a major Mycobacterium tuberculosis outbreak: Retrospective cohort study in a low incidence setting using sparse time-series sampling
Since 1992, Denmark has documented the largest outbreak of tuberculosis in Scandinavia ascribed to a single genotype, termed ‘C2/1112-15’. As of spring 2017, the International Reference Laboratory of Mycobacteriology in Copenhagen has collected and identified isolates from more than a thousand cases belonging to this outbreak via routine MIRU-VNTR typing. Here, we present a retrospective analysis of the C2/1112-15 dataset, based on whole-genome data from a sparse time-series consisting of five randomly selected isolates from each of the 23 years. Even if these data are derived from only 12% of the collected isolates, we have been able to extract important key information, such as mutation rate, conserved single-nucleotide polymorphisms to identify discrete transmission chains, as well as the possible historical origins of the outbreak.

General information
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Reconstruction of the metabolic network of Pseudomonas aeruginosa to interrogate virulence factor synthesis

Virulence-linked pathways in opportunistic pathogens are putative therapeutic targets that may be associated with less potential for resistance than targets in growth-essential pathways. However, efficacy of virulence-linked targets may be affected by the contribution of virulence-related genes to metabolism. We evaluate the complex interrelationships between growth and virulence-linked pathways using a genome-scale metabolic network reconstruction of Pseudomonas aeruginosa strain PA14 and an updated, expanded reconstruction of P. aeruginosa strain PAO1. The PA14 reconstruction accounts for the activity of 112 virulence-linked genes and virulence factor synthesis pathways that produce 17 unique compounds. We integrate eight published genome-scale mutant screens to validate gene essentiality predictions in rich media, contextualize intra-screen discrepancies and evaluate virulence-linked gene distribution across essentiality datasets. Computational screening further elucidates interconnectivity between inhibition of virulence factor synthesis and growth. Successful validation of selected gene perturbations using PA14 transposon mutants demonstrates the utility of model-driven screening of therapeutic targets.
SERS detection of the biomarker hydrogen cyanide from Pseudomonas aeruginosa cultures isolated from cystic fibrosis patients

*Pseudomonas aeruginosa* is the primary cause of chronic airway infections in cystic fibrosis (CF) patients. Persistent infections are seen from the first *P. aeruginosa* culture in about 75% of young CF patients, and it is important to discover new ways to detect *P. aeruginosa* at an earlier stage. The *P. aeruginosa* biomarker hydrogen cyanide (HCN) contains a triple bond, which is utilized in this study because of the resulting characteristic C≡N peak at 2135 cm⁻¹ in a Raman spectrum. The Raman signal was enhanced by surface-enhanced Raman spectroscopy (SERS) on a Au-coated SERS substrate. After long-term infection, a mutation in the patho-adaptive lasR gene can alter the expression of HCN, which is why it is sometimes not possible to detect HCN in the breath of chronically infected patients. Four *P. aeruginosa* reference strains and 12 clinical *P. aeruginosa* strains isolated from CF children were evaluated, and HCN was clearly detected from overnight cultures of all wild type-like isolates and half of the later isolates from the same patients. The clinical impact could be that *P. aeruginosa* infections could be detected at an earlier stage, because daily breath sampling with an immediate output could be possible with a point-of-care SERS device.
A Bacteriophage-Acquired O-Antigen Polymerase (Wzyβ) from P. aeruginosa Serotype O16 Performs a Varied Mechanism Compared to Its Cognate Wzyα

Pseudomonas aeruginosa is a Gram-negative bacterium that produces highly varied lipopolysaccharide (LPS) structures. The O antigen (O-Ag) in the LPS is synthesized through the Wzx/Wzy-dependent pathway where lipid-linked O-Ag repeats are polymerized by Wzy. Horizontal-gene transfer has been associated with O-Ag diversity. The O-Ag present on the surface of serotypes O5 and O16, differ in the intra-molecular bonds, α and β, respectively; the latter arose from the action of three genes in a serotype converting unit acquired from bacteriophage D3, including a β-polymerase (Wzyβ). To further our understanding of O-polymerases, the inner membrane (IM) topology of Wzyβ was determined using a dual phoA-lacZα reporter system wherein random 3’ gene truncations were localized to specific loci with respect to the IM by normalized reporter activities as determined through the ratio of alkaline phosphatase activity to β-galactosidase activity. The topology of Wzyβ developed through this approach was shown to contain two predominant periplasmic loops, PL3 (containing an RXβ motif) and PL4 (having an O-Ag ligase superfamily motif), associated with inverting glycosyltransferase reaction. Through site-directed mutagenesis and complementation assays, residues Arg254, Arg270, Arg272, and His300 were found to be essential for Wzyβ function. Additionally, like-charge substitutions, R254K and R270K, could not complement the wzyβ knockout, highlighting the essential guanidium side group of Arg residues. The O-Ag ligase domain is conserved among heterologous Wzy proteins that produce β-linked O-Ag repeat units. Taking advantage of the recently obtained whole-genome sequence of serotype O16 a candidate promoter was identified. Wzyβ under its native promoter was integrated in the PAO1 genome, which resulted in simultaneous production of α- and β-linked O-Ag. These observations established that members of Wzy-like family consistently exhibit a dual-periplasmic loops topology, and identifies motifs that are plausible to be involved in enzymatic activities. Based on these results, the phage-derived Wzyβ utilizes a different reaction mechanism in the P. aeruginosa host to avoid self-inhibition during serotype conversion.

General information
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Antibiotic combination therapy can select for broad-spectrum multidrug resistance in *Pseudomonas aeruginosa*

Combination therapy with several antibiotics is one strategy that has been applied in order to limit the spread of antimicrobial resistance. We compared the de novo evolution of resistance during combination therapy with the β-lactam ceftazidime and the fluoroquinolone ciprofloxacin with the resistance evolved after single-drug exposure. Combination therapy selected for mutants that displayed broad-spectrum resistance, and a major resistance mechanism was mutational inactivation of the repressor gene mexR that regulates the multidrug efflux operon mexAB–oprM. Deregulation of this operon led to a broad-spectrum resistance phenotype that decreased susceptibility to the combination of drugs applied during selection as well as to unrelated antibiotic classes. Mutants isolated after single-drug exposure displayed narrow-spectrum resistance and carried mutations in the MexCD–OprJ efflux pump regulator gene nfxB conferring ciprofloxacin resistance, or in the gene encoding the non-essential penicillin-binding protein DacB conferring ceftazidime resistance. Reconstruction of resistance mutations by allelic replacement and in vitro fitness assays revealed that in contrast to single antibiotic use, combination therapy consistently selected for mutants with enhanced fitness expressing broad-spectrum resistance mechanisms.
Application of WGS data for O-specific antigen analysis and in silico serotyping of Pseudomonas aeruginosa isolates

Accurate typing methods are required for efficient infection control. The emergence of whole genome sequencing (WGS) technologies has enabled the development of genomics-based methods applicable for routine typing and surveillance of bacterial pathogens. In this study, we developed the Pseudomonas aeruginosa serotyper (PAst) program, which enabled in silico serotyping of P. aeruginosa isolates using WGS data. PAst has been made publically available as a web-service, and aptly facilitate high-throughput serotyping analysis. The program overcomes critical issues such as the loss of in vitro typeability often associated with P. aeruginosa isolates from chronic infections, and quickly determines the serogroup of an isolate based on the sequence of the O-specific antigen (OSA) gene cluster. Here, PAst analysis of 1649 genomes resulted in successful serogroup assignments in 99.27% of the cases. This frequency is rarely achievable by conventional
serotyping methods. The limited number of non-typeable isolates found using PAst was the result of either complete absence of OSA genes in the genomes or the artifact of genomic misassembly. With PAst, P. aeruginosa serotype data can be obtained from WGS information alone. PAst is a highly efficient alternative to conventional serotyping methods in relation to outbreak surveillance of serotype O12 and other high-risk clones, while maintaining backward compatibility to historical serotype data.

General information
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Biofilm as a production platform for heterologous production of rhamnolipids by the non-pathogenic strain *Pseudomonas putida* KT2440

**Background**

Although a transition toward sustainable production of chemicals is needed, the physiochemical properties of certain biochemicals such as biosurfactants make them challenging to produce in conventional bioreactor systems. Alternative production platforms such as surface-attached biofilm populations could potentially overcome these challenges. Rhamnolipids are a group of biosurfactants highly relevant for industrial applications. However, they are mainly produced by the opportunistic pathogen *Pseudomonas aeruginosa* using hydrophobic substrates such as plant oils. As the biosynthesis is tightly regulated in *P. aeruginosa* a heterologous production of rhamnolipids in a safe organism can relieve the production from many of these limitations and alternative substrates could be used.

**Results**

In the present study, heterologous production of biosurfactants was investigated using rhamnolipids as the model compound in biofilm encased *Pseudomonas putida* KT2440. The rhlAB operon from *P. aeruginosa* was introduced into *P. putida* to produce mono-rhamnolipids. A synthetic promoter library was used in order to bypass the normal regulation of rhamnolipid synthesis and to provide varying expression levels of the rhlAB operon resulting in different levels of rhamnolipid production. Biosynthesis of rhamnolipids in *P. putida* decreased bacterial growth rate but stimulated biofilm formation by enhancing cell motility. Continuous rhamnolipid production in a biofilm was achieved using flow cell technology. Quantitative and structural investigations of the produced rhamnolipids were made by ultra performance liquid chromatography combined with high resolution mass spectrometry (HRMS) and tandem HRMS. The predominant rhamnolipid congener produced by the heterologous *P. putida* biofilm was mono-rhamnolipid with two C10 fatty acids.

**Conclusion**

This study shows a successful application of synthetic promoter library in *P. putida* KT2440 and a heterologous biosynthesis of rhamnolipids in biofilm encased cells without hampering biofilm capabilities. These findings expands the possibilities of cultivation setups and paves the way for employing biofilm flow systems as production platforms for biochemicals, which as a consequence of physiochemical properties are troublesome to produce in conventional fermenter setups, or for production of compounds which are inhibitory or toxic to the production organisms.
Curbing the development and spread of 'high risk' bacterial pathogens

MDR (multi-drug Resistant) og XDR (Extensively drug resistant) Pseudomonas aeruginosa clone types spread at alarming rates in hospital environments, and there is a clear need to limit the development and spread of these 'high risk' bacterial pathogens. I will describe our recent efforts to use genomic information to determine the mechanism by which these clone types evolve and spread, and our work on the development a web-based tool that can make identification of high risk clones faster in the clinical microbiology hospital departments. The rationale behind the development of this tool is that faster diagnosis will help to improve containment of the pathogens.

Evolution of metabolic divergence in Pseudomonas aeruginosa during long-term infection facilitates a proto-cooperative interspecies interaction

The effect of polymicrobial interactions on pathogen physiology and how it can act either to limit pathogen colonization or to potentiate pathogen expansion and virulence are not well understood. Pseudomonas aeruginosa and Staphylococcus aureus are opportunistic pathogens commonly found together in polymicrobial human infections. However, we have previously shown that the interactions between these two bacterial species are strain dependent. Whereas P. aeruginosa PAO1, a commonly used laboratory strain, effectively suppressed S. aureus growth, we observed a commensal-like interaction between the human host-adapted strain, DK2-P2M24-2003, and S. aureus. In this study, characterization by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) imaging mass spectrometry (IMS) and mass spectral (MS) molecular networking revealed a significant metabolic divergence between P. aeruginosa PAO1 and DK2-P2M24-2003, which comprised several virulence factors and signaling 4-hydroxy-2-alkylquinoline (HAQ) molecules. Strikingly, a further modulation of the HAQ profile was observed in DK2-P2M24-2003 during interaction with S. aureus, resulting in an area with thickened colony morphology at the P. aeruginosa–S. aureus interface. In addition, we found an HAQ-mediated protection of S. aureus by DK2-P2M24-2003 from the killing effect of tobramycin. Our findings suggest a model where the metabolic divergence manifested in human host-adapted P. aeruginosa is further modulated during interaction with S. aureus and facilitate a proto-cooperative P. aeruginosa–S. aureus relationship.
Evolution of Transcriptional Regulatory Networks in Pseudomonas aeruginosa During Long Time Growth in Human Hosts

Bacteria are remarkable organisms with the capacity to adapt to new environments by remodelling their gene expression profiles. The specific genomic material of any bacterium determines its capacity for any gene regulatory repertoire. However, by evolutionary shaping, these regulatory networks are subjected to forces that allow the bacteria to break genomic constraints, remodel existing regulatory networks, and colonise new environments. While experimental evolution...
studies have documented that global regulators of gene expression are indeed targets for adaptive mutations, it is less clear to which extent these observations relate to natural microbial populations.

The focus of this thesis has been to study how regulatory networks evolve in natural systems. By using a particular infectious disease scenario (human associated persistent airway infections caused by the bacterium Pseudomonas aeruginosa) as a natural model system, the work has focused on characterising a number of mutations in global regulators that are known to provide an adaptive advantage in this specific environment. The aim has been to provide a molecular explanation of the effects of the specific mutations in relation to regulatory network remodelling, and to provide insight into the extent of epistasis and evolutionary dynamics of these systems.

The two studies presented in this thesis specifically deal with single amino acid substitutions or deletions in the sigma factors RpoD, AlgT, and RpoN. Through in vitro techniques, we characterised the direct molecular effects of the sigma factors’ abilities to interact with DNA and the core RNA polymerase (RNAP). By combining this approach with in vivo transcription profile data, Chromatin Immunoprecipitation-sequencing (ChIP-seq) data and artificial regulatory network modifications by in vivo sigma factor overexpression, we were able to investigate how the altered molecule-to-molecule interactions induce rewiring of transcriptional regulatory networks and create unexpected phenotypes.

The results show that through remodelling of the respective regulatory networks, mutations fixed in global regulator genes facilitate the generation of novel phenotypes which again facilitate the shift in life-style of the bacterium from an environmental opportunist pathogen to a human airway specific pathogen. These findings are not only applicable to P. aeruginosa specific studies, but suggest that, on a general level, evolutionary remodelling of regulatory network structures may be the key to ecological success in the wild.

General information
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Genome Sequence of Pseudomonas aeruginosa Strain DK1-NH57388A, a Stable Mucoid Cystic Fibrosis Isolate
Pseudomonas aeruginosa is an important opportunistic pathogen associated with chronic pulmonary infections and mortality in cystic fibrosis (CF) patients. Here, we present the complete genome sequence of stable mucoid P. aeruginosa strain DK1-NH57388A, a CF isolate which has previously been used to establish chronic lung infections in an animal model.

General information
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The extensive use of fossil fuels has a severe influence on the environment. In order to reduce the dependency on these limited resources and to protect the environment, substantial effort is being made to implement renewable resources. One part of this transition is to develop methods for sustainable production of chemicals, which can be achieved by microbial cell factories. The work presented in this PhD thesis elucidates the application of Pseudomonas putida as a microbial cell factory for production of the biosurfactant rhamnolipid. The rhamnolipid production was achieved by heterologous expression of the rhlAB operon from Pseudomonas aeruginosa using a synthetic promoter library in P. putida. Since rhamnolipid production is associated with difficulties in conventional bioreactors, we have used biofilm encased P. putida to circumvent these problems. We show that biofilm can be used as a production platform for continuous production of rhamnolipids. A method for quantitative and qualitative analysis of the produced rhamnolipids was developed based on ultra performance liquid chromatography combined with high resolution mass spectrometry. This enabled detection of low levels of rhamnolipids. The applicability of glycerol as a substrate was also investigated. Since glycerol is a poor substrate, adaptive evolution was made in order to improve the capabilities of P. putida to proliferate on glycerol. The evolved lineages all had significantly increased growth rate, enhanced cell density and reduced lag phase. The genomic alterations were identified by genome sequencing and revealed parallel evolution. Glycerol was also shown to be able to support biofilm growth and as a result of this, it can be used as an alternative substrate for producing biochemicals in conventional and biofilm reactors. The use of biofilm as a production platform and the usage of glycerol as a feedstock show the potential of using microbial cell factories in the transition toward sustainable production of chemicals. Particularly, the applicability of biofilm as a production platform can emerge as a promising alternative for producing toxic biochemicals and for producing biochemicals which are difficult to cope in conventional bioreactors.
Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking

The potential of the diverse chemistries present in natural products (NP) for biotechnology and medicine remains untapped because NP databases are not searchable with raw data and the NP community has no way to share data other than in published papers. Although mass spectrometry (MS) techniques are well-suited to high-throughput characterization of NP, there is a pressing need for an infrastructure to enable sharing and curation of data. We present Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu), an open-access knowledge base for community-wide organization and sharing of raw, processed or identified tandem mass (MS/MS) spectrometry data. In GNPS, crowdsourced curation of freely available community-wide reference MS libraries will underpin improved annotations. Data-driven social-networking should facilitate identification of spectra and foster collaborations. We also introduce the concept of 'living data' through continuous reanalysis of deposited data.
Substantial molecular evolution and mutation rates in prolonged latent Mycobacterium tuberculosis infection in humans

The genome of Mycobacterium tuberculosis (Mtb) of latently infected individuals may hold the key to understanding the processes that lead to reactivation and progression to clinical disease. We report here analysis of pairs of Mtb isolates from putative prolonged latent TB cases. We identified two confirmed cases, and used whole genome sequencing to investigate the mutational processes that occur over decades in latent Mtb. We found an estimated mutation rate between 0.2 and 0.3 over 33 years, suggesting that latent Mtb accumulates mutations at rates similar to observations from cases of active disease.
The evolution of antimicrobial peptide resistance in Pseudomonas aeruginosa is shaped by strong epistatic interactions

Colistin is an antimicrobial peptide that has become the only remaining alternative for the treatment of multidrug-resistant Gram-negative bacterial infections, but little is known of how clinical levels of colistin resistance evolve. We use in vitro experimental evolution and whole-genome sequencing of colistin-resistant Pseudomonas aeruginosa isolates from cystic fibrosis patients to reconstruct the molecular evolutionary pathways open for high-level colistin resistance. We show that the evolution of resistance is a complex, multistep process that requires mutation in at least five independent loci that synergistically create the phenotype. Strong intergenic epistasis limits the number of possible evolutionary pathways to resistance. Mutations in transcriptional regulators are essential for resistance evolution and function as nodes that potentiate further evolution towards higher resistance by functionalizing and increasing the effect of the other mutations. These results add to our understanding of clinical antimicrobial peptide resistance and the prediction of resistance evolution.

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The phenotypic evolution of *Pseudomonas aeruginosa* populations changes in the presence of subinhibitory concentrations of ciprofloxacin

Ciprofloxacin is a widely used antibiotic, in the class of quinolones, for treatment of *Pseudomonas aeruginosa* infections. The immediate response of *P. aeruginosa* to subinhibitory concentrations of ciprofloxacin has been investigated previously. However, the long-term phenotypic adaptation, which identifies the fitted phenotypes that have been selected during evolution with subinhibitory concentrations of ciprofloxacin, has not been studied. We chose an experimental evolution approach to investigate how exposure to subinhibitory concentrations of ciprofloxacin changes the evolution of *P. aeruginosa* populations compared to unexposed populations. Three replicate populations of *P. aeruginosa* PAO1 and its hypermutable mutant ΔmutS were cultured aerobically for approximately 940 generations by daily passages in LB medium with and without subinhibitory concentration of ciprofloxacin and aliquots of the bacterial populations were regularly sampled and kept at -80 °C for further investigations. We investigate here phenotypic changes between the ancestor (50 colonies) and evolved populations (120 colonies/strain). Decreased protease activity and swimming motility, higher levels of quorum-sensing signal molecules and occurrence of mutator subpopulations were observed in the ciprofloxacin-exposed populations compared to the ancestor and control populations. Transcriptomic analysis showed downregulation of the type III secretion system in evolved populations compared to the ancestor population and upregulation of denitrification genes in ciprofloxacin-evolved populations. In conclusion, the presence of antibiotics at subinhibitory concentration in the environment affects bacterial evolution and further studies are needed to obtain insight into the dynamics of the phenotypes and the mechanisms involved.

General information

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Utilization and control of ecological interactions in polymicrobial infections and community-based microbial cell factories

Microbial activities are most often shaped by interactions between co-existing microbes within mixed-species communities. Dissection of the molecular mechanisms of species interactions within communities is a central issue in microbial ecology, and our ability to engineer and control microbial communities depends, to a large extent, on our knowledge of these interactions. This review highlights the recent advances regarding molecular characterization of microbe-microbe interactions that modulate community structure, activity, and stability, and aims to illustrate how these findings have helped us reach an engineering-level understanding of microbial communities in relation to both human health and industrial biotechnology.

Bacteriocin-mediated competition in cystic fibrosis lung infections

Bacteriocins are toxins produced by bacteria to kill competitors of the same species. Theory and laboratory experiments suggest that bacteriocin production and immunity play a key role in the competitive dynamics of bacterial strains. The extent to which this is the case in natural populations, especially human pathogens, remains to be tested. We examined the role of bacteriocins in competition using Pseudomonas aeruginosa strains infecting lungs of humans with cystic fibrosis (CF). We assessed the ability of different strains to kill each other using phenotypic assays, and sequenced their genomes to determine what bacteriocins (pyocins) they carry. We found that (i) isolates from later infection stages inhibited earlier infecting strains less, but were more inhibited by pyocins produced by earlier infecting strains and carried fewer pyocin types; (ii) this difference between early and late infections appears to be caused by a difference in pyocin diversity between competing genotypes and not by loss of pyocin genes within a lineage over time; (iii) pyocin inhibition does not explain why certain strains outcompete others within lung infections; (iv) strains frequently carry the pyocin-killing gene, but not the immunity gene, suggesting resistance occurs via other unknown mechanisms. Our results show that, in contrast to patterns observed in experimental studies, pyocin production does not appear to have a major influence on strain competition during CF lung infections.
Clinical utilization of genomics data produced by the international *Pseudomonas aeruginosa* consortium

The International *Pseudomonas aeruginosa* Consortium is sequencing over 1000 genomes and building an analysis pipeline for the study of *Pseudomonas* genome evolution, antibiotic resistance and virulence genes. Metadata, including genomic and phenotypic data for each isolate of the collection, are available through the International *Pseudomonas* Consortium Database (http://ipcd.ibis.ulaval.ca/). Here, we present our strategy and the results that emerged from the analysis of the first 389 genomes. With as yet unmatched resolution, our results confirm that *P. aeruginosa* strains can be divided into three major groups that are further divided into subgroups, some not previously reported in the literature. We also provide the first snapshot of *P. aeruginosa* strain diversity with respect to antibiotic resistance. Our approach will allow us to draw potential links between environmental strains and those implicated in human and animal infections, understand how patients become infected and how the infection evolves over time as well as identify prognostic markers for better evidence-based decisions on patient care.

General information

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Epistatic Mutations And Unpredictable Phenotypes In Pseudomonas Aeruginosa

*Pseudomonas aeruginosa* is an opportunistic pathogen, able to adapt to stressful environments such as the cystic fibrosis (CF) airways. Adaptation of *P. aeruginosa* to the CF environment is associated with phenotypic changes, such as switch in mucoidy, antibiotic resistance and loss of virulence factors. The phenotypic changes arise from mutations in trans-regulatory elements but are nearly impossible to predict from sequence data alone. Often, the combinatorial effects of few mutations in global regulators give rise to unexpected phenotypes. To understand the epistatic effect and how unexpected phenotypes arise from seemingly unrelated mutations, we have studied two mutations in *P. aeruginosa* transcriptional regulators, sigma factor *rpoD* and *algT*.

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Evolution and adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways: cystic fibrosis as a model system
For centuries evolution has been investigated with an "end-point" approach, through comparisons between species or fossil records. However, to understand processes in general, including evolution, it is highly valuable to observe the dynamics as they unfold, in "realtime". This is possible through laboratory experiments, with a high degree of control and rigour. But to truly understand evolution and the complex mechanisms it deploys, it is necessary to combine the laboratory learnings with investigations of natural systems. Though, this can be tricky. Because of the heterogeneity and constant change of natural environments, the primary obstacle is re-sampling of the same population over time, especially if the...
population is small. Nevertheless, it has been accomplished: Chronic airway infections of cystic fibrosis (CF) patients have offered a unique view into the adaptation and evolution of Pseudomonas aeruginosa to this natural environment, spanning thousands of bacterial generations. Because of the prolonged and persistent infections, they provide a valuable model system for the investigation of evolutionary mechanisms. The main focus of this thesis has been to show the link between evolutionary studies in the CF model system and general evolutionary theories, many of which have been developed from observations of other organisms. This comparison has initially been sought by showing the plausibility of using comprehensive collections of longitudinally sampled single isolates, for their use in evolutionary studies (Study 1). This was done by comparing 5 metagenomes with single isolates from four CF patients, and identifying significant genetic links found within the patient specific P. aeruginosa populations. This evident genetic link was even found for two populations, where a recent patient-to-patient transmission had occurred. Secondly a comprehensive collection of 474 longitudinal single P. aeruginosa isolates from 34 young Danish CF patients was investigated by whole genome sequencing (Study 2). This was done to reconstruct the recent evolutionary history, and identify genes targeted in the initial adaptation to the CF airways. From this analysis we found common clonal lineages among the patients, evidence of patient-to-patient transmission, historic contingencies, and convergent evolution of 52 candidate pathoadaptive genes. By further genome sequencing 26 P. aeruginosa isolates from four Italian CF patients (Study 3), and 35 P. aeruginosa isolates from 12 primary ciliary dyskinesia (PCD) patients (Study 4), we were able to find genetic and phenotypic links across countries and diseases. All three studies (not including the metagenome study) had common clonal lineages and clear overlaps of genetic adaptational patterns. However, the genetic overlap between CF and PCD isolates did not extend to a phenotypic overlap, which indicates that the mucus, which is different in CF patients compared to PCD patients, is a significant selective factor for the evolution and adaptation of P. aeruginosa to these environments. Independently and together the studies presented in this thesis provide new knowledge of adaptation and evolution in both CF and PCD airways. With further characterisation of genetic and phenotypic adaptations it should be possible to translate these results into clinically relevant information, leading to better epidemiological predictions, valuable information with regards to treatment strategies, and perhaps extrapolation of this knowledge to other infection scenarios. OVER ALL: Through the convergence of genetic and phenotypic adaptations observed in CF studies and by linking processes of evolution to these observations, this thesis shows that collections of longitudinal P. aeruginosa isolates from CF patients provide a valuable basis for the study of adaptation and evolution in natural environments.
Genomic Evolution Of The Mdr Serotype O12 Pseudomonas Aeruginosa Clone

Introduction: Since the 1980's the serotype O12 of Pseudomonas aeruginosa has emerged as the predominant serotype in clinical settings and in epidemic outbreaks. These serotype O12 isolates exhibit high levels of resistance to various classes of antibiotics.

Methods: In this study, we explore how the P. aeruginosa LPS biosynthesis gene clusters evolve in the population by investigating the phylogenetic relationship among 83 P. aeruginosa strains and their serotype. In the process we develop a program for in silico serotyping of P. aeruginosa isolates, the P. aeruginosa serotyper (PAst).

Results: While most serotypes were closely linked to the core genome phylogeny we observed horizontal exchange of LPS genes among distinct P. aeruginosa strains. Specifically, we identified a 'serotype island' containing the P. aeruginosa O12 LPS gene cluster and an antibiotic resistance determinant (gyrAC248T) that has been transferred among P. aeruginosa strains. Acquisition and recombination of the 'serotype island' resulted in expression of the O12 serotype in the recipient strains. Conclusions: This observation demonstrate a strong selective advantage for this type of genomic recombination, and suggest that serotype switching in combination with an antibiotic resistance determinant contributed to the dissemination of the O12 serotype in the clinic. This selective advantage coincides with the introduction of fluoroquinolones in the clinic. With the PAst program isolates can be serotyped using WGS data, and dangerous clones like O12 can be identified quickly.

General information
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**phuR intergenic mutation results in pleiotropic effects on global gene expression**

We have previously found a positive selection for promoter mutations in *Pseudomonas aeruginosa* DK2 leading to increased expression of the *phu* (*Pseudomonas* heme utilization) system. By mimicking conditions of the CF airways *in vitro*, we experimentally demonstrated that increased expression of *phuR* confers a growth advantage in the presence of hemoglobin, thus suggesting that *P. aeruginosa* evolves towards iron acquisition from hemoglobin.

**General information**

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**Metabolic adaptation of a human pathogen during chronic infections - a systems biology approach**

Biological systems are complex. When we want to understand biological processes we often need advanced methods to reveal the relationship between genotype and phenotype.

The focus of this thesis has been to extract biological meaningful features from complex data sets and to use mathematical modeling to uncover how human pathogens adapt to the human host. *Pseudomonas aeruginosa* infections in cystic fibrosis patients are used as a model system for understanding these adaptation processes.

The exploratory systems biology approach facilitates identification of important phenotypes and metabolic pathways that are necessary or related to establishment of chronic infections. Archetypal analysis showed to be successful in extracting relevant phenotypes from global gene expression data. Furthermore, genome-scale metabolic modeling showed to be useful in connecting the genotype to phenotype at a systemic level. Particular metabolic subsystems were identified as important for metabolic adaptation in *P. aeruginosa*. One altered metabolic phenotype was connected to a genetic change; a finding that was possible through the systems characterization and which was not identified by classical molecular biology approaches where genes and reactions typically are investigated in a one to one relationship.

This thesis is an example of how mathematical approaches and modeling can facilitate new biological understanding and provide new surprising ideas to important biological processes.

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**Novel Path Towards Colistin Resistance In *Pseudomonas Aeruginosa* During Chronic Infection Involves Polymorphisms In Uncharacterized Glycosyltransferase Gene**

Introduction: Antibiotic resistance development in the gram-negative bacterium *Pseudomonas aeruginosa* is an increasing problem. The effect of colistin, one of the few last resort drugs commonly given to cystic fibrosis (CF) patients, is
dependent on the lipopolysaccharide (LPS) structure. We have identified a novel gene cluster, which is involved in colistin susceptibility in chronically infecting *P. aeruginosa* strains. The gene cluster contains two uncharacterized glycosyltransferases and a gene of unknown function. During chronic infection of CF patients one of the glycosyltransferase genes is prone to mutation. Methods: The glycosyltransferase single nucleotide polymorphism (SNP) was reverted to the reference genotype in a clinical isolate and in parallel introduced into the laboratory reference strain PAO1 to provide a clear background for mutational analysis. We evaluated minimal inhibitory concentration by microbroth dilution, virulence in an amoebae model and LPS structure by visualization in a silver-stained gel. Results: Reversion of the SNP to reference genotype resulted in increased colistin susceptibility, reduced virulence in an amoebae model and altered LPS structure. The results indicate that this glycosyltransferase polymorphism is needed for the clinical strain to be fully virulent. However, introducing the SNP into PAO1 did not result in altered phenotypes. These results reveal this uncharacterized glycosyltransferase as a novel in vivo path to colistin resistance by LPS modification. Conclusions: Colistin resistance development in vivo occurs via multiple paths. Here a novel pathway for the development of colistin resistance was described. It involves mutations in a hitherto uncharacterized glycosyltransferase.

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**Rnaseq As A Method To Study Microbial Interactions Arising In The Cystic Fibrosis Airways**

Introduction: In previous studies from our laboratory, a *Pseudomonas aeruginosa* lineage, named DK2, has been identified and characterized as highly successful, transmissible and persistent over four decades in cystic fibrosis (CF) patients. This lineage underwent substantial phenotypic and genetic changes over time and therefore provides a unique opportunity to explore the impact of those adaptational pathways on its ability to interact with other pathogenic bacteria such as *Staphylococcus aureus*, a pathogen frequently co-infecting the CF airways. Methods: We have used a novel method to study interspecies interactions between a CF isolate (2003) from the DK2 lineage and a wild-type *S. aureus* JE2. We grew both strains in mono or co-culture on LB agar, harvested RNA from the colonies after a 24-hour period. Subsequently we performed RNA-seq for the different samples. The data were then compared in a pairwise mode to isolate the transcriptomic profiles for each species. The most differentially expressed genes from both species were validated using real-time quantitative PCR. Results: Interestingly, the greatest expression change was observed in *S. aureus*, where large clusters of genes associated with virulence were differentially expressed, compared with the monoculture condition, while the *P. aeruginosa* DK2 response was much more discrete with isolated genes differentially regulated rather than whole operons or clusters. Conclusions: According to our data, *S. aureus* would display reduced virulence in the presence of an adapted *P. aeruginosa* DK2 clone, possibly as a consequence of the multiple hostile forces DK2 encountered over time during its long-term adaptation to the CF airways.

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**Substantial Molecular Evolution In Prolonged Latent Mycobacterium Tuberculosis Infections In Humans**

Introduction: Despite its central role as a reservoir for active tuberculosis disease (TB), latent *Mycobacterium tuberculosis* (*Mtbc*) infections and the underlying persistence mechanisms are poorly understood. The *Mtbc* genome in latently infected individuals may hold the key to understanding the processes that lead to reactivation and progression to clinical disease. Methods: We studied genomic relationships among 14 isolates of *Mtbc* from historical and recent Danish clinical strain collections, spanning more than three decades, to investigate 6 putative cases of *Mtbc* reactivation, inferred from IS6110 profiles. Single-nucleotide polymorphism (SNPs) patterns were analyzed to identify true cases of TB re-activation, as well
as the underlying mutational patterns. Results: Two parallel cases of latent TB reactivation were identified. We found an average mutation rate of 0.2 – 0.3 over 33 years, as well as evidence for distinct processes such as oxidative damage or natural selection having contributed to mutation accumulation. Conclusions: Our study shows that distinct processes can shape Mtb genomes during latent infection. Most importantly, we document substantial molecular evolution of Mtb over three decades, with mutation rates similar to observations from cases of active disease. Our study thus emphasizes the importance of identifying and controlling latent cases.

The widespread multi-drug-resistant serotype O12 Pseudomonas aeruginosa clone emerged through concomitant horizontal transfer of serotype antigen and antibiotic resistance gene clusters

The O-specific antigen (OSA) in Pseudomonas aeruginosa lipopolysaccharide is highly varied by sugar identity, side chains, and bond between O-repeats. These differences classified P. aeruginosa into 20 distinct serotypes. In the past few decades, O12 has emerged as the predominant serotype in clinical settings and outbreaks. These serotype O12 isolates exhibit high levels of resistance to various classes of antibiotics. Here, we explore how the P. aeruginosa OSA biosynthesis gene clusters evolve in the population by investigating the association between the phylogenetic relationships among 83 P. aeruginosa strains and their serotypes. While most serotypes were closely linked to the core genome phylogeny, we observed horizontal exchange of OSA biosynthesis genes among phylogenetically distinct P. aeruginosa strains. Specifically, we identified a "serotype island" ranging from 62 kb to 185 kb containing the P. aeruginosa O12 OSA gene cluster, an antibiotic resistance determinant (gyrAC248T), and other genes that have been transferred between P. aeruginosa strains with distinct core genome architectures. We showed that these genes were likely acquired from an O12 serotype strain that is closely related to P. aeruginosa PA7. Acquisition and recombination of the "serotype island" resulted in displacement of the native OSA gene cluster and expression of the O12 serotype in the recipients. Serotype switching by recombination has apparently occurred multiple times involving bacteria of various genomic backgrounds. In conclusion, serotype switching in combination with acquisition of an antibiotic resistance determinant most likely contributed to the dissemination of the O12 serotype in clinical settings. Infection rates in hospital settings by multidrug-resistant (MDR) Pseudomonas aeruginosa clones have increased during the past decades, and serotype O12 is predominant among these epidemic strains. It is not known why the MDR phenotype is associated with serotype O12 and how this clone type has emerged. This study shows that evolution of MDR O12 strains involved a switch from an ancestral O4 serotype to O12. Serotype switching was the result of horizontal transfer and genetic recombination of lipopolysaccharide (LPS) biosynthesis genes originating from an MDR taxonomic outlier P. aeruginosa strain. Moreover, the recombination event also resulted in acquisition of antibiotic resistance genes. These results impact
on our understanding of MDR outbreak strain and serotype evolution and can potentially assist in better monitoring and prevention.

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**Working in the biomedical engineering domain: opportunities and challenges**

**General information**

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Diversity Generation in Evolving Microbial Populations

Organisms have evolved and diversified since the beginning of life. Although, generation and maintenance of diversity within ecosystems has been a central concern in ecology and evolutionary biology, little is known of the evolutionary processes driving diversification. Especially, diversification in relation to chronic infection is a major concern as high population diversity has been predicted to result in survival and persistence of the infecting microbe. Therefore, understanding within-host dynamics and population diversification is necessary for optimal diagnosis and therapeutic treatment.

Chronic Pseudomonas aeruginosa infections in the airways of patients with cystic fibrosis (CF) offer opportunities to study bacterial evolution and adaptation in natural environments. Significantly phenotypic and genomic changes of P. aeruginosa have been observed during chronic infection. While P. aeruginosa diversity has been documented in contemporary respiratory specimens, it is less clear to what extent within-patient diversity contributes to the overall population structure and whether the population is geographically or homogeneously distributed throughout the airways. The focus of this thesis has been to get a better understanding of how bacterial populations adapt to new, complex and heterogeneous environments with multiple selective pressures over long periods, and to analyse diversification during this adaptation. Using the P. aeruginosa chronic infection as a model system, and by combining bacterial genome sequencing, phenotypic profiling and unique sampling materials which included clonal bacterial isolates sampled for more than 4 decades from chronically infected CF patients, we were able to investigate the diversity generation of the clinical important and highly successful P. aeruginosa DK1 clone type during chronic airway infection in CF patients.

We show here that diversification of P. aeruginosa DK1 occurs through the emergence of coexisting subpopulations with distinct phenotypic and genomic features and demonstrate that this diversification was a result of niche specialization as each subpopulation colonized separate geographical niches. This highly complex population diversity was observed to be stably maintained during long-term evolution. Before diversification of the DK1 clone, a regulatory mutation was found to be fixed in the population causing alteration of multiple phenotypes representing the chronic stage phenotype.

Often chronic CF infections are polyclonal and therefore we investigated the population dynamics in a patient polyclonal infected with both DK1 and DK2. We demonstrated that diversification was affected by the presence of other clones; interaction between the two clones resulted in horizontal DNA transfer that contributed to the observed population diversity by creating a novel strain DK1/2 found to persist in the CF airways.

These data indicate that spatial compartmentalization and transfer of DNA between infecting microbes can cause generation and maintenance of population diversity of infecting pathogens. Furthermore, fine-tuning of global regulatory networks by modification of transcriptional regulators has fundamental roles in successful adaptation of P. aeruginosa to the CF environment.

Environmental heterogeneity drives within-host diversification and evolution of Pseudomonas aeruginosa.

Within-host pathogen evolution and diversification during the course of chronic infections is of importance in relation to therapeutic intervention strategies, yet our understanding of these processes is limited. Here, we investigate intracranial population diversity in P. aeruginosa during chronic airway infections in cystic fibrosis patients. We show the evolution of a diverse population structure immediately after initial colonization, with divergence into multiple distinct sublineages that coexisted for decades and occupied distinct niches. Our results suggest that the spatial heterogeneity in CF airways plays a major role in relation to the generation and maintenance of population diversity and emphasize that a single isolate in sputum may not represent the entire pathogen population in the infected individual. A more complete understanding of the evolution of distinct clonal variants and their distribution in different niches could have positive implications for efficient therapy.
Staphylococcus aureus Alters Growth Activity, Autolysis, and Antibiotic Tolerance in a Human Host-Adapted Pseudomonas aeruginosa Lineage.

Interactions among members of polymicrobial infections or between pathogens and the commensal flora may determine disease outcomes. Pseudomonas aeruginosa and Staphylococcus aureus are important opportunistic human pathogens and are both part of the polymicrobial infection communities in human hosts. In this study, we analyzed the in vitro interaction between S. aureus and a collection of P. aeruginosa isolates representing different evolutionary steps of a dominant lineage, DK2, that have evolved through decades of growth in chronically infected patients. While the early adapted P. aeruginosa DK2 strains outcompeted S. aureus during coculture on agar plates, we found that later P. aeruginosa DK2 strains showed a commensal-like interaction, where S. aureus was not inhibited by P. aeruginosa and the growth activity of P. aeruginosa was enhanced in the presence of S. aureus. This effect is mediated by one or more extracellular S. aureus proteins greater than 10 kDa, which also suppressed P. aeruginosa autolysis and prevented killing by clinically relevant antibiotics through promoting small-colony variant (SCV) formation. The commensal interaction was abolished with S. aureus strains mutated in the agr quorum sensing system or in the SarA transcriptional virulence regulator, as well as with strains lacking the proteolytic subunit, ClpP, of the Clp protease. Our results show that during evolution of a dominant cystic fibrosis lineage of P. aeruginosa, a commensal interaction potential with S. aureus has developed.
Within-host evolution of Pseudomonas aeruginosa reveals adaptation toward iron acquisition from hemoglobin.

Pseudomonas aeruginosa airway infections are a major cause of mortality and morbidity of cystic fibrosis (CF) patients. In order to persist, P. aeruginosa depends on acquiring iron from its host, and multiple different iron acquisition systems may be active during infection. This includes the pyoverdine siderophore and the Pseudomonas heme utilization (phu) system. While the regulation and mechanisms of several iron-scavenging systems are well described, it is not clear whether such systems are targets for selection during adaptation of P. aeruginosa to the host environment. Here we investigated the within-host evolution of the transmissible P. aeruginosa DK2 lineage. We found positive selection for promoter mutations leading to increased expression of the phu system. By mimicking conditions of the CF airways in vitro, we experimentally demonstrate that increased expression of phuR confers a growth advantage in the presence of hemoglobin, thus suggesting that P. aeruginosa evolves toward iron acquisition from hemoglobin. To rule out that this adaptive trait is specific to the DK2 lineage, we inspected the genomes of additional P. aeruginosa lineages isolated from CF airways and found similar adaptive evolution in two distinct lineages (DK1 and PA clone C). Furthermore, in all three lineages, phuR promoter mutations coincided with the loss of pyoverdine production, suggesting that within-host adaptation toward heme utilization is triggered by the loss of pyoverdine production. Targeting heme utilization might therefore be a promising strategy for the treatment of P. aeruginosa infections in CF patients. IMPORTANCE Most bacterial pathogens depend on scavenging iron within their hosts, which makes the battle for iron between pathogens and hosts a hallmark of infection. Accordingly, the ability of the opportunistic pathogen Pseudomonas aeruginosa to cause chronic infections in cystic fibrosis (CF) patients also depends on iron-scavenging systems. While the regulation and mechanisms of several such iron-scavenging systems have been well described, not much is known about how the within-host selection pressures act on the pathogens' ability to acquire iron. Here, we investigated the within-host evolution of P. aeruginosa, and we found evidence that P. aeruginosa during long-term infections evolves toward iron acquisition from hemoglobin. This adaptive strategy might be due to a selective loss of other iron-scavenging mechanisms and/or an increase in the availability of hemoglobin at the site of infection. This information is relevant to the design of novel CF therapeutics and the development of models of chronic CF infections.
Within-host evolution of *Pseudomonas aeruginosa* toward iron acquisition from hemoglobin in polymicrobial CF infections

Bacterial pathogens require iron to survive and colonize a human host but their access to free iron is often limited by iron-withholding process where free iron is bound by proteins such as hemoglobin. Although most pathogens have developed tactics to acquire iron from host proteins, little is known about how evolutionary processes modulate bacterial iron acquisition systems in chronic, polymicrobial infections where interspecies competition for limited iron could be an evolutionary driver.

### General information

**State:** Published  
**Organisations:** Department of Systems Biology, Infection Microbiology  
**Authors:** Khademi, S. M. H. (Intern), Marvig, R. L. (Intern), Pedersen, S. D. (Intern), Jelsbak, L. (Intern)  
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Within_host_evolution_of_Pseudomonas_aeruginosa_toward_iron_acquisition_from_hemoglobin_in_polymicrobial_CF_infections.publishers.pdf  
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### Archetypal analysis of diverse *Pseudomonas aeruginosa* transcriptomes reveals adaptation in cystic fibrosis airways

**BACKGROUND:** Analysis of global gene expression by DNA microarrays is widely used in experimental molecular biology. However, the complexity of such high-dimensional data sets makes it difficult to fully understand the underlying biological features present in the data. The aim of this study is to introduce a method for DNA microarray analysis that provides an intuitive interpretation of data through dimension reduction and pattern recognition. We present the first “Archetypal Analysis” of global gene expression. The analysis is based on microarray data from five integrated studies of *Pseudomonas aeruginosa* isolated from the airways of cystic fibrosis patients. RESULTS: Our analysis clustered samples into distinct groups with comprehensible characteristics since the archetypes representing the individual groups are closely related to samples present in the data set. Significant changes in gene expression between different groups identified adaptive changes of the bacteria residing in the cystic fibrosis lung. The analysis suggests a similar gene expression pattern between isolates with a high mutation rate (hypermutators) despite accumulation of different mutations for these isolates. This suggests positive selection in the cystic fibrosis lung environment, and changes in gene expression...
for these isolates are therefore most likely related to adaptation of the bacteria. CONCLUSIONS: Archetypal analysis
succeeded in identifying adaptive changes of P. aeruginosa. The combination of clustering and matrix factorization made it
possible to reveal minor similarities among different groups of data, which other analytical methods failed to identify. We
suggest that this analysis could be used to supplement current methods used to analyze DNA microarray data.

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Authors: Thøgersen, J. C. (Intern), Mørup, M. (Intern), Pedersen, S. D. (Intern), Molin, S. (Intern), Jelsbak, L. (Intern)
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Draft Genome Sequences of Pseudomonas aeruginosa B3 Strains Isolated from a Cystic Fibrosis Patient Undergoing Antibiotic Chemotherapy

Pseudomonas aeruginosa frequently establishes chronic infections in the airways of patients suffering from cystic fibrosis (CF). Here, we report the draft genome sequences of four P. aeruginosa B3 strains isolated from a chronically infected CF patient undergoing antibiotic chemotherapy.

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Evolution and Pathoadaptation of Pseudomonas aeruginosa in Cystic Fibrosis Patients

Molecular and mechanistic understanding of evolution is essential for our ability to comprehend the development of life on Earth. Life appeared around 4 billion years ago, and has ever since adapted and diversified through the process of evolution. The focus of this thesis has been to increase our understanding of how bacteria evolve and genetically adapt in a natural environment. In particular we sought to identify the genes that are targeted by mutation to optimize fitness in a given environment, and to understand the evolutionary mechanisms that govern the genetic change. Pseudomonas aeruginosa is the dominating pathogen of chronic airway infections in patients with cystic fibrosis (CF), and the bacterial long-term persistence in CF hosts involves mutation and selection of genetic variants with increased fitness in the CF airways. We performed a retrospective study of the P. aeruginosa DK2 clone type, which is a transmissible clone isolated from chronically infected Danish CF patients over a period of 38 years. Whole-genome analysis of DK2 isolates enabled a fine-grained reconstruction of the recent evolutionary history of the DK2 lineage and an identification of bacterial genes targeted by mutations to optimize pathogen fitness. The identification of such pathoadaptive genes gives new insight into how the pathogen evolves under the selective pressures of the host immune system and drug therapies. Furthermore, isolates with increased rates of mutation (hypermutator phenotype) emerged in the DK lineage. While this phenotype may accelerate evolution, we also showed that hypermutators display differential mutagenesis of certain genes which enable them to follow alternative evolutionary pathways. Overall, our study identifies genes important for bacterial adaptation to a human host environment and provides insight into the different mutational mechanisms that govern the adaptive genetic changes.

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Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts
The genetic basis of bacterial adaptation to a natural environment has been investigated in a highly successful Pseudomonas aeruginosa lineage (DK2) that evolved within the airways of patients with cystic fibrosis (CF) for more than 35 y. During evolution in the CF airways, the DK2 lineage underwent substantial phenotypic changes, which correlated with temporal fixation of specific mutations in the genes mucA (frame-shift), algT (substitution), rpoN (substitution), lasR (deletion), and rpoD (in-frame deletion), all encoding regulators of large gene networks. To clarify the consequences of these genetic changes, we moved the specific mutations, alone and in combination, to the genome of the reference strain PAO1. The phenotypes of the engineered PAO1 derivatives showed striking similarities with phenotypes observed among the DK2 isolates. The phenotypes observed in the DK2 isolates and PAO1 mutants were the results of individual, additive and epistatic effects of the regulatory mutations. The mutations fixed in the σ factor encoding genes algT, rpoN, and rpoD caused minor changes in σ factor activity, resulting in remodeling of the regulatory networks to facilitate generation of unexpected phenotypes. Our results suggest that adaptation to a highly selective environment, such as the CF airways, is a highly dynamic and complex process, which involves continuous optimization of existing regulatory networks to match the fluctuations in the environment.

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Organisations: Department of Systems Biology, Center for Systems Microbiology
Genome Analysis of a Transmissible Lineage of Pseudomonas aeruginosa Reveals Pathoadaptive Mutations and Distinct Evolutionary Paths of Hypermutators.

Genome sequencing of bacterial pathogens has advanced our understanding of their evolution, epidemiology, and response to antibiotic therapy. However, we still have only a limited knowledge of the molecular changes in in vivo evolving bacterial populations in relation to long-term, chronic infections. For example, it remains unclear what genes are mutated to facilitate the establishment of long-term existence in the human host environment, and in which way acquisition of a hypermutator phenotype with enhanced rates of spontaneous mutations influences the evolutionary trajectory of the pathogen. Here we perform a retrospective study of the DK2 clone type of *P. aeruginosa* isolated from Danish patients suffering from cystic fibrosis (CF), and analyze the genomes of 55 bacterial isolates collected from 21 infected individuals over 38 years. Our phylogenetic analysis of 8,530 mutations in the DK2 genomes shows that the ancestral DK2 clone type spread among CF patients through several independent transmission events. Subsequent to transmission, sub-lineages evolved independently for years in separate hosts, creating a unique possibility to study parallel evolution and identification of genes targeted by mutations to optimize pathogen fitness (pathoadaptive mutations). These genes were related to antibiotic resistance, the cell envelope, or regulatory functions, and we find that the prevalence of pathoadaptive mutations correlates with evolutionary success of co-evolving sub-lineages. The long-term coexistence of both normal and hypermutator populations enabled comparative investigations of the mutation dynamics in homopolymeric sequences in which hypermutators are particularly prone to mutations. We find a positive exponential correlation between the length of the homopolymer and its likelihood to acquire mutations and identify two homopolymer-containing genes preferentially mutated in hypermutators. This homopolymer facilitated differential mutagenesis provides a novel genome-wide perspective on the different evolutionary trajectories of hypermutators, which may help explain their emergence in CF infections.

**General information**

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Organisations: Department of Systems Biology, Infection Microbiology
Authors: Marvig, R. L. (Intern), Johansen, H. K. (Intern), Molin, S. (Intern), Jelsbak, L. (Intern)
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BFI (2015): BFI-level 2
Most knowledge about evolutionary adaptation has been gained from experimental evolution studies, in which organisms have been allowed to evolve under simple, well-defined conditions in the laboratory. While these studies have provided novel insight into the fundamental processes of evolutionary adaptation, it is less clear to which extent the observations can be generalized to natural systems, in which organisms evolve in complex heterogeneous environments.

The focus of this thesis has been to explore different aspects of evolutionary adaptation of bacterial populations evolving in natural environments. The model system used for these investigations has been long-term chronic airway infections in Cystic fibrosis (CF) patients caused by the opportunistic pathogen Pseudomonas aeruginosa. Using a systems biology approach, we have monitored the adaptive development of the clinically important P. aeruginosa DK2 clone lineage during 200,000 generations of evolution in the CF airways from its entrance in the clinic in the 1970’ies until the end of 2010.

Genetic analysis showed that the DK2 lineage between 1973 and 2007 accumulated mutations in a near-linear manner with an overall genomic signature of negative selection. Phenotypic profiling (gene expression and catabolic performance) showed that major phenotypic changes occurred in the DK2 lineage during the early years until 1979, after which only marginal phenotypic changes could be observed in and between isolates. Through the use of genetic reconstructions it was shown that many of the phenotypic changes were caused by mutations in genes encoding regulators of large regulatory networks in P. aeruginosa. Moreover, it was shown that the combination of mutations gave rise to unexpected CF-adaptive phenotypes such as increased tolerance to antibiotics as well as a conditional mucoid phenotype which was induced only upon exposure to CF-associated stress conditions such as high osmolarity and anaerobic conditions.

Appearance of a constitutive mucoid phenotype was discovered during the late stages of the DK2 colonization in a specific CF patient. The mucoid DK2 isolates emerged in connection with a prolonged period of non-compliance to antibiotic treatment and correlated with a permanent increase in the inflammatory response. Genetic analysis shows that several mucoid variants evolved in parallel by distinct mutational pathways; one of these pathways included fixation of mutations in the rpoD gene encoding the principle sigma factor σ70.

The findings presented in this thesis provide insight into the genetic mechanisms and evolutionary processes that shape the adaptation of bacteria colonizing complex natural environments. Increased knowledge of the evolutionary trajectories...
of pathogens may lead to significant improvements in the future treatment of patients suffering from severe chronic infections.

Typing of Pseudomonas aeruginosa from hemorrhagic pneumonia in mink (Neovison vison)

Hemorrhagic pneumonia in mink (Neovison vison) is caused by Pseudomonas aeruginosa and is an acute and fatal disease in farmed mink. Earlier work has demonstrated that some outbreaks of hemorrhagic pneumonia are caused by pathogenic strains while most outbreaks are caused by local strains. The objective of this study was to determine the genetic and geographical relationship among outbreaks of hemorrhagic pneumonia by pulsed field gel electrophoresis typing of P. aeruginosa isolates. Furthermore, chosen isolates were typed by a commercial genotyping method based on single nucleotide polymorphisms (SNPs) and compared to a larger dataset of human and environmental origin. The bacterial isolates were obtained from diagnostic samples from 2002-2009 and contained 164 isolates from 95 outbreaks on 90 farms. Our results show that most outbreaks of hemorrhagic pneumonia in mink are caused by distinct strains of P. aeruginosa. We also identified related P. aeruginosa strains which, together with two prevalent but unrelated clones, caused one third of the outbreaks of hemorrhagic pneumonia supporting the sparse literature on this subject. None of the SNP typed strains were identified in a large dataset of human and environmental origin.
Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an evolutionary perspective.

The airways of patients with cystic fibrosis (CF) are nearly always infected with many different microorganisms. This environment offers warm, humid and nutrient-rich conditions, but is also stressful owing to frequent antibiotic therapy and the host immune response. Pseudomonas aeruginosa is commonly isolated from the airways of patients with CF, where it most often establishes chronic infections that usually persist for the rest of the lives of the patients. This bacterium is a major cause of mortality and morbidity and has therefore been studied intensely. Here, we discuss how P. aeruginosa evolves from a state of early, recurrent intermittent colonization of the airways of patients with CF to a chronic infection state, and how this process offers opportunities to study bacterial evolution in natural environments. We believe that such studies are valuable not only for our understanding of bacterial evolution but also for the future development of new therapeutic strategies to treat severe chronic infections.
Deletion and acquisition of genomic content during early stage adaptation of Pseudomonas aeruginosa to a human host environment

Adaptation of bacterial pathogens to a permanently host-associated lifestyle by means of deletion or acquisition of genetic material is usually examined through comparison of present-day isolates to a distant theoretical ancestor. This limits the resolution of the adaptation process. We conducted a retrospective study of the dissemination of the P. aeruginosa DK2 clone type among patients suffering from cystic fibrosis, sequencing the genomes of 45 isolates collected from 16 individuals over 35 years. Analysis of the genomes provides a high-resolution examination of the dynamics and mechanisms of the change in genetic content during the early stage of host adaptation by this P. aeruginosa strain as it adapts to the cystic fibrosis (CF) lung of several patients. Considerable genome reduction is detected predominantly through the deletion of large genomic regions, and up to 8% of the genome is deleted in one isolate. Compared with in vitro estimates the resulting average deletion rates are 12- to 36-fold higher. Deletions occur through both illegitimate and homologous recombination, but they are not IS element mediated as previously reported for early stage host adaptation. Uptake of novel DNA sequences during infection is limited as only one prophage region was putatively inserted in one isolate, demonstrating that early host adaptation is characterized by the reduction of genomic repertoire rather than acquisition of novel functions. Finally, we also describe the complete genome of this highly adapted pathogenic strain of P. aeruginosa to strengthen the genetic basis, which serves to help our understanding of microbial evolution in a natural environment.
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ISI indexed (2012): ISI indexed yes
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Scopus rating (2010): SJR 2.775 SNIP 1.551
Web of Science (2010): Indexed yes
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Web of Science (2009): Indexed yes
BFI (2008): BFI-level 2
Scopus rating (2008): SJR 2.495 SNIP 1.322
Web of Science (2008): Indexed yes
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Scopus rating (2006): SJR 2.451 SNIP 1.517
Web of Science (2006): Indexed yes
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Web of Science (2003): Indexed yes
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**Evolution and diversification of Pseudomonas aeruginosa in the paranasal sinuses of cystic fibrosis children have implications for chronic lung infection**

The opportunistic pathogen Pseudomonas aeruginosa is a frequent colonizer of the airways of patients suffering from cystic fibrosis (CF). Depending on early treatment regimens, the colonization will, with high probability, develop into chronic infections sooner or later, and it is important to establish under which conditions the switch to chronic infection takes place. In association with a recently established sinus surgery treatment program for CF patients at the Copenhagen CF Center, colonization of the paranasal sinuses with P. aeruginosa has been investigated, paralleled by sampling of sputum from the same patients. On the basis of genotyping and phenotypic characterization including transcription profiling, the diversity of the P. aeruginosa populations in the sinuses and the lower airways was investigated and compared. The observations made from several children show that the paranasal sinuses constitute an important niche for the colonizing bacteria in many patients. The paranasal sinuses often harbor distinct bacterial subpopulations, and in the early colonization phases there seems to be a migration from the sinuses to the lower airways, suggesting that
independent adaptation and evolution take place in the sinuses. Importantly, before the onset of chronic lung infection, lineage with mutations conferring a large fitness benefit in CF airways such as mucA and lasR as well as small colony variants and antibiotic-resistant clones are part of the sinus populations. Thus, the paranasal sinuses potentially constitute a protected niche of adapted clones of P. aeruginosa, which can intermittently seed the lungs and pave the way for subsequent chronic lung infections.
Evolutionary dynamics of pseudomonas aeruginosa in CF

General information
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Organisations: Department of Systems Biology, Center for Systems Microbiology, Novo Nordisk Foundation Center for Biosustainability, Bacterial Cell Factories, Copenhagen University Hospital
Authors: Molin, S. (Intern), Jelsbak, L. (Intern), Johansen, H. (Forskerdatabase), Hoiby, N. (Ekstern)
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Scopus rating (2012): SJR 0.917 SNIP 0.923 CiteScore 1.93
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Scopus rating (2011): SJR 0.883 SNIP 0.915 CiteScore 1.93
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BFI (2009): BFI-level 1
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Web of Science (2009): Indexed yes
BFI (2008): BFI-level 1
Scopus rating (2008): SJR 1.091 SNIP 0.948
Scopus rating (2007): SJR 0.981 SNIP 0.987
Web of Science (2007): Indexed yes
Scopus rating (2006): SJR 0.942 SNIP 1.033
Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria

Accurate strain identification is essential for anyone working with bacteria. For many species, multilocus sequence typing (MLST) is considered the "gold standard" of typing, but it is traditionally performed in an expensive and time-consuming manner. As the costs of whole-genome sequencing (WGS) continue to decline, it becomes increasingly available to scientists and routine diagnostic laboratories. Currently, the cost is below that of traditional MLST. The new challenges will be how to extract the relevant information from the large amount of data so as to allow for comparison over time and between laboratories. Ideally, this information should also allow for comparison to historical data. We developed a Web-based method for MLST of 66 bacterial species based on WGS data. As input, the method uses short sequence reads from four sequencing platforms or preassembled genomes. Updates from the MLST databases are downloaded monthly, and the best-matching MLST alleles of the specified MLST scheme are found using a BLAST-based ranking method. The sequence type is then determined by the combination of alleles identified. The method was tested on preassembled genomes from 336 isolates covering 56 MLST schemes, on short sequence reads from 387 isolates covering 10 schemes, and on a small test set of short sequence reads from 29 isolates for which the sequence type had been determined by traditional methods. The method presented here enables investigators to determine the sequence types of their isolates on the basis of WGS data. This method is publicly available at www.cbs.dtu.dk/services/MLST.

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Web of Science (2015): Indexed yes
Mutations in 23S rRNA Confer Resistance against Azithromycin in Pseudomonas aeruginosa
The emergence of antibiotic-resistant Pseudomonas aeruginosa is an important concern in the treatment of long-term airway infections in cystic fibrosis patients. In this study, we report the occurrence of azithromycin resistance among clinical P. aeruginosa DK2 isolates. We demonstrate that resistance is associated with specific mutations (A2058G, A2059G, and C2611T in Escherichia coli numbering) in domain V of 23S rRNA and that introduction of A2058G and C2611T into strain PAO1 results in azithromycin resistance.
Aspergillus triggers phenazine production in *Pseudomonas aeruginosa*

Objectives: *Pseudomonas aeruginosa* is an opportunistic human pathogen, commonly infecting cystic fibrosis (CF) patients. Aspergilli, especially *Aspergillus fumigatus*, are also frequently isolated from CF patients. Our aim was to examine the possible interaction between *P. aeruginosa* and different Aspergillus species. Methods: A suspension of fungal spores was streaked onto WATM agar plates. After 24 hours incubation at 37 °C, a *P. aeruginosa* overnight culture was streaked out perpendicular to the fungal streak. The plates were incubated at 37 °C for five days, examined and plugs were extracted for HPLC-DAD and HPLC-DAD-MS analysis. Results: *P. aeruginosa* PAO1 suppressed growth of *A. fumigatus*, *A. niger*, *A. flavus*, *A. oryzae*, *A. terreus* and *Emericella nidulans*. HPLC and HPLC-DAD-MS results showed an increase in phenazine-1-carboxylic acid and phenazine-1-carboxamide production by *P. aeruginosa* in the contact area of *A. niger*, *A. flavus*, *A. oryzae*, but not *A. fumigatus*. In addition, other metabolites with UV chromophores similar to the phenazines were only found in the contact zone between Aspergillus and Pseudomonas. No change in secondary metabolite profiles were seen for the Aspergilli, when comparing with or without the presence of Pseudomonas. Conclusion: All Aspergilli tested, with the exception of *A. fumigatus*, triggered the upregulation of phenazine-1-carboxamide and phenazine-1-carboxylic acid production by *P. aeruginosa*. Surprisingly no changes in secondary metabolite profiles were detected in any of the Aspergilli.

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specific adaption strategies during the early stage infections while certain essential adaptations are evolved in parallel during the chronic infections.

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Evolutionary dynamics of bacteria in a human host environment

Laboratory evolution experiments have led to important findings relating organism adaptation and genomic evolution. However, continuous monitoring of long-term evolution has been lacking for natural systems, limiting our understanding of these processes in situ. Here we characterize the evolutionary dynamics of a lineage of a clinically important opportunistic bacterial pathogen, Pseudomonas aeruginosa, as it adapts to the airways of several individual cystic fibrosis patients over 200,000 bacterial generations, and provide estimates of mutation rates of bacteria in a natural environment. In contrast to predictions based on in vitro evolution experiments, we document limited diversification of the evolving lineage despite a highly structured and complex host environment. Notably, the lineage went through an initial period of rapid adaptation caused by a small number of mutations with pleiotropic effects, followed by a period of genetic drift with limited phenotypic change and a genomic signature of negative selection, suggesting that the evolving lineage has reached a major adaptive peak in the fitness landscape. This contrasts with previous findings of continued positive selection from long-term in vitro evolution experiments. The evolved phenotype of the infecting bacteria further suggests that the opportunistic pathogen has transitioned to become a primary pathogen for cystic fibrosis patients.
Microbial ecology and adaptation in cystic fibrosis airways

Chronic infections in the respiratory tracts of cystic fibrosis (CF) patients are important to investigate, both from medical and from fundamental ecological points of view. Cystic fibrosis respiratory tracts can be described as natural environments harbouring persisting microbial communities with Pseudomonas aeruginosa as a dominant pathogen. Various factors...
contribute to the complexity of this ecosystem, including community composition, dynamics and interactions, as well as heterogeneous distribution and fluctuation of components of the immune system, antibiotics and nutrients. All these elements constitute the selective forces that drive the evolution of the microbes after they migrate from the outer environment to human airways. Pseudomonas aeruginosa adapts to the new environment through genetic changes and exhibits a special lifestyle in chronic CF airways. Understanding the persistent colonization of microbial pathogens in CF patients in the context of ecology and evolution will expand our knowledge of the pathogenesis of chronic infections and improve therapeutic strategies.
Suppression of Aspergillus by Pseudomonas aeruginosa

Objectives: Cystic fibrosis patients are commonly infected by Pseudomonas aeruginosa, but Aspergilli are also frequently isolated. Our aim was to examine the possible interaction between P. aeruginosa and different Aspergillus. Methods: A suspension of 10^6 fungal spores/ml was streaked onto WATM culture plates. After 24 hours incubation at 37 °C, a P. aeruginosa overnight culture diluted to 10^8 CFU/ml was streaked out perpendicular to the fungal streak. The plates were incubated at 37 °C for 5 days, examined and plugs were extracted for HPLC and LC-DAD-MS analysis. Results: P. aeruginosa PAO1 suppressed growth of A. fumigatus, A. niger, A. flavus, A. oryzae, A. terreus and E. nidulans. HPLC and LC-DAD-MS results showed an increase in phenazine-1-carboxylic acid and phenazine-1-carboxamide production by P. aeruginosa in the contact area of Aspergillus. Different quinolones were also identified, here among 2-heptyl-3-hydroxy-4-quinolone (PQS). An unidentified green pseudomonas compound was also observed. Interestingly the P. aeruginosa mutant rpoN was unable to suppress A. fumigatus, but suppressed A. flavus, A. oryzae and A. niger. However several other P. aeruginosa mutants suppressed A. fumigatus including flif, pilA, lasR, PVDA, PQSC and rhlA mutants indicating that phenazines may be involved in the suppressed growth of A. fumigatus. All pseudomonas mutants suppressed A. oryzae, A. niger and A. flavus. Conclusions: An increase in phenazine production by P. aeruginosa may contribute to the ability of P. aeruginosa to suppress different Aspergilli. Especially phenazines seem to play a role, while other factors such as motility, rhamnolipid and alginate production do not seem to be involved.

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Early adaptive developments of Pseudomonas aeruginosa after the transition from life in the environment to persistent colonization in the airways of human cystic fibrosis hosts

Pseudomonas aeruginosa is an opportunistic pathogen ubiquitous to the natural environment but with the capability of moving to the host environment. Long-term infection of the airways of cystic fibrosis patients is associated with extensive genetic adaptation of P. aeruginosa, and we have studied cases of the initial stages of infection in order to characterize the early adaptive processes in the colonizing bacteria. A combination of global gene expression analysis and phenotypic
characterization of longitudinal isolates from cystic fibrosis patients revealed well-known characteristics such as conversion to a mucoid phenotype by mucA mutation and increased antibiotic resistance by nfxB mutation. Additionally, upregulation of the atu operon leading to enhanced growth on leucine provides a possible example of metabolic optimization. A detailed investigation of the mucoid phenotype uncovered profound pleiotropic effects on gene expression including reduction of virulence factors and the Rhl quorum sensing system. Accordingly, mucoid isolates displayed a general reduction of virulence in the Caenorhabditis elegans infection model, altogether suggesting that the adaptive success of the mucoid variant extends beyond the benefits of alginate overproduction. In the overall perspective the global phenotype of the adapted variants appears to place them on paths in direction of fully adapted strains residing in long-term chronically infected patients.

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In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections

The growth dynamics of bacterial pathogens within infected hosts are a fundamental but poorly understood feature of most infections. We have focused on the in situ distribution and growth characteristics of two prevailing and transmissible Pseudomonas aeruginosa clones that have caused chronic lung infections in cystic fibrosis (CF) patients for more than 20 years. We used fluorescence in situ hybridization (FISH) directly on sputum specimens to examine the spatial distribution of the infecting P. aeruginosa cells. Mucoid variants were present in sputum as cell clusters surrounded by an extracellular matrix, whereas nonmucoid variants were present mainly as dispersed cells. To obtain estimates of the growth rates of P. aeruginosa in CF lungs, we used quantitative FISH to indirectly measure growth rates of bacteria in sputum samples (reflecting the in vivo lung conditions). The concentration of rRNA in bacteria isolated from sputa was measured and correlated with the rRNA contents of the same bacteria growing in vitro at defined rates. The results showed that most cells were actively growing with doubling times of between 100 and 200 min, with some growing even faster. Only a small stationary-phase subpopulation seemed to be present in sputa. This was found for both mucoid and nonmucoid variants despite their different organizations in sputum. The results suggest that the bacterial population may be confronted with selection forces that favor optimized growth activities. This scenario constitutes a new perspective on the adaptation and evolution of P. aeruginosa during chronic infections in CF patients in particular and on long-term infections in general.
Complete genome sequence of the myxobacterium Sorangium cellulosum
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Molecular epidemiology and dynamics of Pseudomonas aeruginosa populations in lungs of cystic fibrosis patients

The ability to establish lifelong persistent infections is a fundamental aspect of the interactions between many pathogenic microorganisms and their mammalian hosts. One example is chronic lung infections by the opportunistic pathogen Pseudomonas aeruginosa in cystic fibrosis (CF) patients. This infection process is associated with extensive genetic adaptation and microevolution of the infecting bacteria. Through investigations of P. aeruginosa populations and infection dynamics in a group of CF patients followed at the Danish CF Clinic in Copenhagen, we have identified two distinct and dominant clones that have evolved into highly successful colonizers of CF patient airways. A significant component of the evolutionary success of these two clones has been their efficient transmissibility among the CF patients. The two clones have been present and transmitted among different CF patients for more than 2 decades. Our data also suggest that the P. aeruginosa population structure in the CF patient airways has been influenced by competition between different clones and that the two dominant clones have been particularly competitive within the lungs, which may add to their overall establishment success. In contrast, we show that adaptive traits commonly associated with establishment of chronic P. aeruginosa infections of CF patients, such as transition to the mucoid phenotype and production of virulence factors, play minor roles in the ability of the two dominant clones to spread among patients and cause long-term chronic infections. These findings suggest that hitherto-unrecognized evolutionary pathways may be involved in the development of successful and persistent P. aeruginosa colonizers of CF patient lungs.
In response to starvation, Myxococcus xanthus initiates a developmental program that results in the formation of spore-filled, multicellular fruiting bodies. Many developmentally regulated genes in M. xanthus are transcribed from sigma(54) promoters, and these genes require enhancer-binding proteins. Here we report the finding of an unusual group of 12 genes encoding sigma(54)-dependent enhancer-binding proteins containing a forkhead-associated (FHA) domain as their N-terminal sensory domain. FHA domains in other proteins recognize phosphothreonine residues. An insertion mutation in one of these genes, Mx4885, caused a cell autonomous aggregation and sporulation defect. In-frame deletion mutants...
showed that the FHA domain is necessary for proper Mx4885 function. The altered pattern of developmental gene expression in the mutant implied that Mx4885 is on the pathway of response to the morphogenetic C-signal. Immunoblots specific for C-signa I and FruA imply that the site of Mx4885 action is downstream of FruA synthesis on the C-signal transduction pathway. Mx4885 may help to coordinate the level of intracellular phosphorylated FruA (FruA-P) with the level of C-signal displayed on the signal donor cell. Because FHA domains respond to phosphothreonine-containing proteins, these results suggest a regulatory link to the abundant Ser/Thr protein kinases in M. xanthus.
σ54 enhancer binding proteins and Myxococcus xanthus fruiting body development

A search of the M1 genome sequence, which includes 97% of the Myxococcus xanthus genes, identified 53 sequence homologs of σ54-dependent enhancer binding proteins (EBPs). A DNA microarray was constructed from the M1 genome that includes those homologs and 318 other M. xanthus genes for comparison. To screen the developmental program with this array, an RNA extract from growing cells was compared with one prepared from developing cells at 12 h. Previous reporter studies had shown that M. xanthus has initiated development and has begun to express many developmentally regulated genes by 12 h. The comparison revealed substantial increases in the expression levels of 11 transcription factors that may respond to environmental stimuli. Six of the 53 EBP homologs were expressed at significantly higher levels at 12 h of development than during growth. Three were previously unknown genes, and they were inactivated to look for effects on fruiting body development. One knockout mutant produced fruiting bodies of abnormal shape that depended on the composition of the medium.
Formation of spatial patterns of cells from a mass of initially identical cells is a recurring theme in developmental biology. The dynamics that direct pattern formation in biological systems often involve morphogenetic cell movements. An example is fruiting body formation in the gliding bacterium Myxococcus xanthus in which an unstructured population of identical cells rearranges into an asymmetric, stable pattern of multicellular fruiting bodies in response to starvation. Fruiting body formation depends on changes in organized cell movements from swarming to aggregation. The aggregation process is induced and orchestrated by the cell-surface associated 17 kDa C-signal protein. C-signal transmission depends on direct contact between cells. Evidence suggests that C-signal transmission is geometrically constrained to cell ends and that productive C-signal transmission only occurs when cells engage in end-to-end contacts. Here, we review recent progress in the understanding of the pattern formation process that leads to fruiting body formation. Gliding motility in M xanthus involves two polarly localized gliding machines, the S-machine depends on type IV pili and the A-machine seems to involve a slime extrusion mechanism. Using time-lapse video microscopy the gliding motility parameters controlled by the C-signal have been identified. The C-signal induces cells to move with increased gliding speeds, in longer gliding intervals and with decreased stop and reversal frequencies. The combined effect of the C-signal dependent changes in gliding motility behaviour is an increase in the net-distance travelled by a cell per minute. The identification of the motility parameters controlled by the C-signal in combination with the contact-dependent C-signal transmission mechanism have allowed the generation of a qualitative model for C-signal induced aggregation. In this model, the directive properties of the C-signal are a direct consequence of the contact-dependent signal-transmission mechanism, which is a local event involving direct contact between cells that results in a global organization of cells. This pattern formation process does not depend on a diffusible substance. Rather it depends on a cell-surface associated signal to direct the cells appropriately.

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Coupling gene expression and multicellular morphogenesis during fruiting body formation in Myxococcus xanthus

A recurring theme in morphogenesis is the coupling of the expression of genes that drive morphogenesis and the morphogenetic process per se. This coupling ensures that gene expression and morphogenesis are carried out in synchrony. Morphogenesis of the spore-filled fruiting bodies in Myxococcus xanthus illustrates this coupling in the construction of a multicellular structure. Fruiting body formation involves two stages: aggregation of cells into mounds and the position-specific sporulation of cells that have accumulated inside mounds. Developmental gene expression propels these two processes. In addition, gene expression in individual cells is adjusted according to their spatial position. Progress in the understanding of the cell surface-associated C-signal is beginning to reveal the framework of an intercellular signalling system that allows the coupling of gene expression and multicellular morphogenesis. Accumulation of the C-signal is tightly regulated and involves transcriptional activation of the csgA gene and proteolysis of the full-length CsgA protein to produce the shorter cell surface-associated 17 kDa C-signal protein. The C-signal induces aggregation, sporulation and developmental gene expression at specific thresholds. The ordered increase in C-signalling levels, in combination with the specific thresholds, allows the C-signal to induce these three processes in the correct temporal order. The contact-dependent C-signal transmission mechanism, in turn, guarantees that C-signalling levels reflect the spatial position of individual cells relative to other cells and, thus, allows the cells to decode their spatial position during morphogenesis. By this mechanism, individual cells can tailor their gene expression profile to one that matches their spatial position. In this scheme, the molecular device that keeps gene expression in individual cells in register with morphogenesis is the C-signalling system, and the morphological structure, which is assessed, is the spatial position of individual cells relative to that of other cells.
Pattern formation by a cell surface-associated morphogen in Myxococcus xanthus

In response to starvation, an unstructured population of identical Myxococcus xanthus cells rearranges into an asymmetric, stable pattern of multicellular fruiting bodies. Central to this pattern formation process are changes in organized cell movements from swarming to aggregation. Aggregation is induced by the cell surface-associated C-signal. To understand how aggregation is accomplished, we have analyzed how C-signal modulates cell behavior. We show that C-signal induces a motility response that includes increases in transient gliding speeds and in the duration of gliding intervals and decreases in stop and reversal frequencies. This response results in a switch in cell behavior from an oscillatory to a unidirectional type of behavior in which the net-distance traveled by a cell per minute is increased. We propose that the C-signal-dependent regulation of the reversal frequency is essential for aggregation and that the remaining C-signal-dependent changes in motility parameters contribute to aggregation by increasing the net-distance traveled by starving cells per minute. In our model for symmetry-breaking and aggregation, C-signal transmission is a local event involving direct contacts between cells that results in a global organization of cells. This pattern formation mechanism does not require a diffusible substance or other actions at a distance. Rather it depends on contact-induced changes in motility behavior to direct cells appropriately.
Pattern formation: fruiting body morphogenesis in Myxococcus xanthus

When Myxococcus xanthus cells are exposed to starvation, they respond with dramatic behavioral changes. The expansive swarming behavior stops and the cells begin to aggregate into multicellular fruiting bodies. The cell-surface-associated C-signal has been identified as the signal that induces aggregation. Recently, several of the components in the C-signal transduction pathway have been identified and behavioral analyses are beginning to reveal how the C-signal...
modulates cell behavior. Together, these findings provide a framework for understanding how a cell-surface-associated morphogen induces pattern formation.
The cell-surface associated, intercellular C-signal induces behavioral changes in individual Myxococcus xanthus cells during fruiting body morphogenesis

Fruiting body formation in Myxococcus xanthus depends on ordered changes in cell movements from swarming to aggregation in response to starvation. We show that appropriately starved individual cells change behavior during fruiting body formation. Specifically, from the time of initiation of aggregation, individual wild-type cells began to move with increased gliding speeds, the duration of the mean gliding interval increased, and the stop frequency decreased whereas the duration of the mean stop interval and the reversal frequency remained unchanged. Mutants lacking the cell surface-associated, intercellular C-signal (csgA mutants) failed to aggregate. Likewise, appropriately starved individual csgA cells did not change their behavior during development. In the absence of other cell–cell interactions, the motility defect of individual csgA cells was corrected in a time- and concentration-dependent manner after C-signaling was reestablished by exogenous MalE-CsgA protein. The C-signal-induced stimulation of motility depended on the cytoplasmic Frz signal transduction system. We propose that C-signal instructs cells to move with high speed and low stop and reversal frequencies into aggregation centers during development.

General information
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