Screening for virulence inhibiting compounds against enteropathogenic *Escherichia coli*

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Summary

Traditional antibiotic therapies have undeniably revolutionized our world, yet they carry limitations and new approaches are needed. Adverse effects are caused by the broad-spectrum action antibiotics as it typically also disrupts beneficial microbial communities. Furthermore, pathogens have developed numerous resistance mechanisms, increasingly compromising the efficacy of these medications. Addressing this challenge could be achieved by developing drugs capable of selectively targeting pathogenic physiological processes, while sparing non-pathogenic microbes.

This doctoral thesis presents a comprehensive investigation into scholarly work on preventing bacterial virulence and presents research done towards discovering small molecule inhibitors of the translocated intimin receptor (Tir), a critical virulence factor involved in enteropathogenic *Escherichia coli* (EPEC) pathogenesis. The translocated intimin receptor plays a pivotal role in the pathogenesis of EPEC, contributing to the formation of so called attaching and effacing (A/E) lesions on host epithelial cells. Despite its significance as a therapeutic target, the development of small-molecule inhibitors against Tir has not been successful. This study employs a multidisciplinary approach combining computational virtual screening methods and experimental high throughput screening to attempt to identify novel small molecules capable of disrupting Tir function.

The presented research consists of three distinct projects. Initially, a previously described microscopy method was expanded into an automated screening technique. Subsequently, an *in silico* virtual screening campaign was conducted using molecular docking to sift through extensive compound libraries for potential small molecule inhibitors of Tir. Seventy-two compounds suggested by virtual screening underwent experimental testing via high throughput screening assays. All resulting hits underwent rigorous assessment for artifactual signals and false positives using cell and bacterial viability assays and ultimately no true actives were identified. Lastly, fractions isolated from cultures of marine actinobacteria were tested using a panel of virulence-related screening assays. Two of these fractions exhibited reduced virulence-based assay readouts. Following isolation, identification and dereplication suggested that one of these fractions, from a *Rhodococcus* strain, likely contains an antimicrobial peptide, while the other fraction, from a *Kocuria* strain. potentially consists of two larger phospholipid compounds.

In sum, this thesis contributes to the progress of drug discovery endeavors aimed at combating bacterial virulence factors. The discoveries outlined herein set the stage for future inquiries into drug development targeting Tir and underscore the constrained efficacy of conventional virtual screening and phenotype-based high-throughput screening approaches in expediting the identification of antivirulence compounds for EPEC.

Tiivistelmä

Antibiootit ovat kiistatta mullistaneet maailmaa, mutta niiden käyttöön liittyy ongelmia, ja uusia hoitomuotoja tarvitaan. Laajakirjoisen vaikutuksensa vuoksi, antibiootit aiheuttavat haittavaikutuksia esimerkiksi elimistön ja ympäristön mikrobiyhteisöihin. Tämän lisäksi useat sairautta aiheuttavat bakteerit ovat tulleet vastustuskykyisiksi antibiooteille heikentäen näin niiden tehoa. Tätä ongelmaa voidaan pyrkiä ratkaisemaan kehittämällä lääkeaineita, joiden vaikutukset kohdentuisivat suoraan sairautta aiheuttaviin fysiologisiin prosesseihin, sen sijaan että ne pyrkivät eliminoimaan kaikkia bakteereja.

Työssä tarkastellaan viimeisimpiä tutkimuslöydöksiä bakteerien virulenssin estämisessä ja esitetään menetelmiä löytää pienimolekyylisiä yhdisteitä estämään translokoidun intimiinireseptorin (Tir) toimintaa. Tir on merkittävä virulenssitekijä, joka liittyy keskeisesti enteropatogeenisen *Escherichia coli*-bakteerin (EPEC) kykyyn aiheuttaa sairautta. Tir vaikuttaa keskeisesti EPECin aiheuttamien leesioiden muodostumiseen isäntänsä epiteelisoluissa. Huolimatta sen keskeisestä roolista sairauden aikaan saamisessa, lääkkeitä Tir-proteiinia vastaan ei ole toistaiseksi onnistuttu kehittämään. Tutkimuksissa käytettiin monitieteistä lähestymistapaa, yhdistäen esimerkiksi laskennallisen sekä virtuaaliseulonnan menetelmiä että kokeellista tehoseulontaa puusien Tir-estäjien löytämiseksi.

Tutkimus koostuu kolmesta erillisestä projektista. Ensimmäisessä osassa aiemmin kuvattu mikroskopiamenetelmä laajennettiin automaattiseksi seulontatekniikaksi. Seuraavaksi suoritettiin tietokoneavusteinen virtuaalinen seulontakampanja, jossa käytettiin molekyylitelakointeja seulomaan yhdistekirjastoja potentiaalisten Tir-estäjien löytämiseksi. Mallinnuksesta valittiin 72 yhdistettä tehoseulontaan. Tästä tutkimuksesta saatuja tuloksia tarkasteltiin kriittisesti mahdollisten virheellisten löytöjen välttämiseksi, ja lopulta todellisia aktiivisia yhdisteitä ei löytynyt. Kolmannessa projektissa, merellisten aktinobakteerien kasvustoista eristettyjä fraktioita testattiin usealla eri virulenssia tutkivalla menetelmällä. Näistä fraktioista kaksi saivat aikaan mitattavia muutoksia. Fraktioiden tarkempi tutkiminen viittasi siihen, että toinen näistä on peräisin *Rhodococcus*-kannasta ja sisältää todennäköisesti antimikrobisen peptidin, kun taas toinen fraktio, *Kocuria*-kannasta, sisältää todennäköisesti kaksi suurempaa fosfolipidia.

Työssä esitetyt tulokset edistävät bakteerien virulenssitekijöitä vastaan suunnattujen lääkekehityksen pyrkimyksiä. Tässä esitetyt löydökset luovat pohjan tuleville tutkimuksille, jotka kohdistuvat Tir-intimiini-interaktioon, ja korostavat perinteisen virtuaalisen seulonnan ja fenotyyppipohjaisten seulonnan rajallisuutta tähän tarkoitukseen.

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List of original publications

This thesis is based on the following 3 publications, which are referred in the text by their roman numerals (I, II, III):

I. Pylkkö, T., Ilina, P. & Tammela, P. Development and validation of a high-content screening assay for inhibitors of enteropathogenic *E. coli* adhesion. *Journal of Microbiological Methods* 106201 (2021)
II. Pylkkö, T., Tomašič, T., Poso, A. & Tammela, P. Virtual Screening Assisted Search for Inhibitors of the Translocated Intimin Receptor of Enteropathogenic *Escherichia Coli*. *Chembiochem* e202300638 (2024)
III. Pylkkö T, Schneider YKH, Rämä T, Andersen JH, Tammela P. Bioprospecting of inhibitors of EPEC virulence from metabolites of marine actinobacteria from the Arctic Sea. Front Microbiol. 2024 Aug 30;15:1432475.

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Author's contributions

I. Tuomas Pylkkö participated in the design of the study and executed all the experimental work. He also wrote all the code used in the study for the robotics, data reduction from images and formal analysis of the results. He also wrote the manuscript and participated in editing and revision with the co-authors.

II. Tuomas Pylkkö participated in the design of the study, performed virtual screening for two of the three models, all the empirical work, collection of images, data reduction, and formal analysis of the results. Tihomir Tomašič created one of the three models. Tuomas Pylkkö also wrote the manuscript and participated in editing and revision with the co-authors.

III. The collection and the optimization of the culture conditions were performed by colleagues at UiT. Tuomas Pylkkö designed the study. Tuomas Pylkkö extracted, fractionated, freeze dried the cultures, and performed all the empirical experiments. Tuomas Pylkkö also collected all the data and analyzed it. Yannik K. Schneider performed chromatography-based isolation, dereplication and analysis of the mass spectrometry features of the compounds identified as active by Tuomas Pylkkö. Tuomas Pylkkö also wrote the manuscript and participated in editing and revision with the co-authors.

Abbreviations and acronyms

A/E	Attaching and effacing
EPEC	Enteropathogenic Eschirichia coli
LEE	Locus of enterocyte effacement
Tir	Translocated intimin receptor
IBD	Intimin binding domain
ECD	Extracellular domain
FAS	Fluorescent actin staining
T3SS	The type three secretion system
PDB	Protein Data Bank
NMR	Nuclear magnetic resonance
BFP	Bundle forming pilus
PPI	Protein protein interaction
PFA	Paraformaldehyde
MEM	Minimum Essential Medium
HPLC	High performance liquid chromatography

1 Introduction

The discovery of antibiotic chemical entities to combat infectious diseases represents the pinnacle achievement in contemporary medicine, ushering in a healthcare revolution that has profoundly impacted the lives of individuals in industrialized nations (Kardos & Demain, 2011). In the era preceding antibiotics, even seemingly minor infections by today's standards posed grave peril, often culminating in fatality. An early example is that of Albert Alexander, one of the first patients treated with penicillin, who, despite near-complete recovery, tragically succumbed to an infection due to the limited supply of the breakthrough drug at the time (Wainwright 1990). Presently, antibiotics not only serve as curative agents for diseases but also assume indispensable roles in domains such as dentistry, surgery, and food production.

Nevertheless, the emergence of widespread resistance to antimicrobial agents and the acknowledged adverse effects associated with broad-spectrum antibiotics have reached a point where the usefulness of these treatments is threatened (Alanis 2005; Resistance 2019). Antibiotic discovery has noticeably decelerated, while antimicrobial resistance presents an escalating issue (World Health Orginization 2022). Although stewardship and control programs offer a lot of mitigation, the imperative for novel therapeutic approaches in the face of this challenge is indisputable. All possible approaches and ideas available will be needed.

This conundrum has prompted scholarly inquiry into alternative modalities for treating bacterial infections. One such approach entails the exploration of virulence-modifying compounds that have the capacity to mitigate infectious diseases without lethal impact on pathogens. It is commonly posited that this approach may be less detrimental to commensal (health promoting) bacteria while exerting less selection pressure for resistance among pathogens (Baron 2010; Defoirdt 2016; Buroni and Chiarelli 2020). Achieving this objective requires a high-level comprehension of virulence mechanisms and the ways to develop interventions to disrupt them – this encapsulates the essence of what we call the "antivirulence approach."

It is important to realize that out of the estimated one trillion bacterial phylotypes in existence, no more than approximately one hundred have been characterized as human pathogens (Curtis, Sloan, and Scannell 2002). This presents a significant quandary for the field of antimicrobial drug development, not solely due to the absence of a comprehensive theory of bacterial virulence and the mechanisms involved, but also because numerous microorganisms play pivotal roles in maintaining human and environmental health, not to mention, that much of what microbes actually achieve in ecosystems at large is for the most part unknown. Pathogenicity appears to be the exception, not a rule. Consequently, conventional antibiotic therapies invariably yield adverse effects due to their indiscriminate effect on other bacteria within the patient and the environment at large. Some of these effects are only now beginning to be understood with the rise of microbiome and environment related metagenomics projects *et cetera* (Rappuoli et al. 2023).

So, given that the detrimental impacts caused by pathogens predominantly stem from their application of virulence-inducing biomolecules, an intriguing question arises for biosciences: Can precisely targeted pharmaceutical interventions effectively mitigate these pathogenic effects without inducing substantial collateral damage? Could the specific problem of virulence be targeted instead of microbial cells? In the subsequent review of the current literature, we delve into recent research and discoveries into alternative strategies for

combating bacterial infections. Through an exploration of these emerging methodologies, I want to illuminate potential avenues for confronting the evolving menace of antibiotic resistance, in particular in the case of EPEC. In particular, we will look at a specific virulence factor pair, intimin and the translocated intimin receptor (Tir) and how they have been approached from the perspective of boh fundamental research and drug development.

2 Literature review

2.1 Short prelude to bacterial virulence and how can it be mitigated

According to Martin Heiddegger, genuinely philosophical questions exist that typically do not have straight answers, but it is nevertheless valuable to ask these as the act of asking itself generates other important questions that can guide us in theorizing and thinking (Heidegger 2001). In the context of this work, such a fundamental question would be: what exactly is virulence; the phenomenon that we aim to prevent? Preferably we would want to have a comprehensive theory with explanatory power within the context of evolution and it to be helpful in the design of drug interventions. Unfortunately, understanding on this topic is limited with many questions remaining unanswered and the putative explanatory theoretical frameworks yet to be tested by experiment and thus, we need to pursue drug development without such a comprehensive explanatory framework. Pertinent questions follow: Why do a select few bacterial strains cause human disease? Why do many pathogens appear to have a tritagonistic origin? What drives them to harm their host, crucial for their own survival? What advantages does this behavior confer, especially in light of the metabolic costs of carrying additional genetic material in their chromosomes? Lastly, why is it often that about 1% of carriers (hosts) exhibit pathogenicity or symptoms? The future will require a deep understanding of where being a health benefiting or benign commensal ends and pathogenicity begins, to be able to address the illness causing nature of the human microbiome.

Nevertheless, in brief, virulence refers to a harm-causing effect of a microorganism, a component of that microbiome. This effect is largely determined by host-pathogen interactions and external circumstances in such a way that 1) occasionally microorganisms genetically capable of virulence *do not* cause illness, and 2) at times host defense reactions themselves can cause seemingly inappropriate levels of harm. From the perspective of the microorganism involved, for virulence to be possible, a set of biological properties are required. Stanley Falkow lists the following necessary capabilities for a microorganism to be capable of causing virulence (Falkow and Margolis 2009) (Table 1).

Table 1. A list of requirements for virulence from Margolis and Falkow (2009) and their appearance in enteropathogenic *E. coli*.

Property	Examples of implementation in EPEC
1. Acquire virulence genes	tRNA sites and plasmids
2. Sense the environment	two-component systems
3. Switch virulence genes on and off	thermoregulation, quorum sensing based regulation of e.g. LEE.
4. Move to the site of infection	pili, flagella, adhesins
5. Become established	microcolonies / niche construction / manipulation of cytoskeleton with intimin and its receptor
6. Acquire nutrients	siderophores and nanotubes
7. Survive stress	pedestal/microcolony niche
8. Avoid the host's immune system	pedestal/microcolony niche, effectors
9. Subvert host cytoskeleton and signaling pathways	virulence factors
10. Disseminate to other sites and/or hosts (in some cases)	retract adhesins, actin-based motility
11. Be transmitted to a new susceptible host	diarrhea-induced pollution via e.g. SGLT1

Many of the above-listed functions are in fact related to niche construction, for example the cell-adhered microcolony in the case of EPEC. It can therefore be reasoned that any successful manner to interfere with these could lead to an asymptomatic existence of EPEC in the intestine or at least decrease or interfere with virulence. It is clear that all of these phenomena have been studied with the aims of learning how they work and how they could be interfered with in a pharmacotherapy. It is not entirely inconceivable that working interventions could be designed without understanding the underlying mechanisms entirely, but at the same time it seems that most successful drug interventions are based on auxiliary fundamental research.

2.2.1 Various explanatory theories of virulence

So, what exactly distinguishes pathogens from non-pathogens? And why do pathogens cause illness? Stanley Falkow and Elisa Margolis lists four long held hypotheses of virulence, which have been named (i) the conventional wisdom, (ii) epidemiological selection, (iii) coincidental evolution, (iv) short-sighted, within-host evolution (Falkow and Margolis 2009).

They point out that none of these have been subjected to falsification attempts with standard scientific methods, although there is convincing indirect evidence and arguments for most of them.

The first of these, the conventional wisdom states that virulence is a consequence of the relatively recent association between a parasite and its new host. As this association progresses, natural selection acting on the host-parasite system may either result in 1) the extinction of one of the participating species, or 2) the evolution towards symbiotic or mutualistic relationships. It is then argued that given that neither the pathogen nor the host under study are extinct, option number two must have occurred. Many bacteria responsible for human morbidity and mortality have been acquired from other species relatively recently, particularly following the emergence of agriculture, and some continue to be acquired in this manner (Anderson and May 1982). Among these zoonotic (and protozoonotic) bacterial infections are diseases such as the plague, tuberculosis, Legionnaires' disease, botulism, anthrax, brucellosis, tularemia, Rocky Mountain spotted fever, cholera, and other diarrheal illnesses (Falkow and Margolis 2009). Enteropathogenic *E. coli* (EPEC) have been reported in dozens of other species, but it is not clear what the significance of this is (McDougall et al. 2023). The main reservoir for a very similar human pathogen, EHEC, is believed to be cattle.

And decreased virulence over time does seem to happen with many pathogens (Cressler et al. 2016), but it is easy to see why such a simple theory would run into problems, and indeed the rest of the hypotheses can be interpreted as modified versions of the same that take these issues into account. Typical problems of coincidentality, nestedness, and granularity in evolution arise. So, for example, while virulence may evolve in whatever direction in order to maximize transmission, that same evolution can also be affected by other trade-offs of stronger effects. Nestedness occurs when, for example, eukaryotic parasites predate on bacteria while bacteriophages infect them from within, leaving open the possibility that e.g. phage based virulence genes may be increasing survival of bacteria from predation by protozoa (and thus the survival of phage genes) despite simultaneously incurring costs on the eukaryotic human host cells. Furthermore, the effect that is under evolution in the explanation may be part or subtype of a larger effect that is also under evolution (nested) or it may be unclear where the unit under evolution begins or ends (resolution or granularity).

According to the epidemiological hypothesis, the evolution of virulence could be explained if the virulence effects themselves facilitate transmission. This is particularly relevant for many intestinal pathogens, which spread via the fecal-oral route (i.e. are food and water borne). In such cases, the manifestation of virulence as diarrhea may enhance transmission via the following increased pathogen pollution of the surrounding water and food. This hypothesis suggests that the transmission advantage conferred by diarrhea outweighs the potential reduction in transmission caused by host mortality. These kinds of mechanisms also probably play in infections via vectors like ticks, since the death of the organism (i.e. a plague infected rodent) will cause the vectors (ticks) to move to a new host to allow for the pathogen (plague) to spread. Under this framework it may make sense for enhanced virulence to evolve for intestinal pathogens.

Coincidental evolution, in turn, is the hypothesis that bacterial products responsible for the morbidity and/or mortality of the host, virulence determinants, evolved in response to selection for some function other than virulence. It has been argued, though not formally demonstrated, that the virulence caused by *Helicobacter pylori* in some individuals offers absolutely no advantage to *H. pylori*, but the following chronic gastric inflammation, tissue

damage and increased risk of gastric cancer follow from the constant immune reaction and are entirely coincidental. This also points to an important theoretical point: that virulence is an interaction between pathogen and host. Similarly, any property increasing transmission in one context may cause virulence as a side effect in another context and vice versa. A concrete example of such different needs in different contexts is that enteric pathogens spend a significant amount of time outside of the host where very different laws of fitness pervade than within the host. Indeed, many generations of the pathogen may live without ever coming into contact with it. And thus when enteropathogenic *E. coli* are cultured in temperatures below typical endotherm (35-39 °C) temperatures, they do not express virulence factors (Umanski, Rosenshine, and Friedberg 2002). The existence of these kinds of mechanisms, e.g. regulation of genes, of course on the other hand, is exactly what needs to be researched and analyzed in order to be able to design pathoblockers and antivirulence approaches, as it may be possible to pharmaceutically manipulate the metabolism, gene regulation or signaling required by the pathogen for a virulent lifestyle.

Despite this apparent uncertainty in the possibilities in reasons for virulence, it seems clear to us that in the case of enteropathogens there are multiple different mechanisms of infection, but the outcomes are often similar, leading to symptoms such as diarrhea. This likely increases their transmission from host to host. We can see that the determining factor is not the serotype *per se*, but existing virulence inducing genetic material and that this material does not induce virulence without reaching a specific niche or site (and possible other external conditions need to be valid). Virulence may occur at such a low level as to not cause immediate symptoms. For example, it has been noticed in the last decades that so-called atypical EPEC strains are very prevalent, but cause less severe symptoms (Kralicek et al. 2022). These may nevertheless be unwanted, as it has been suspected that EPEC infection may be a risk factor for colorectal cancer (Magdy et al. 2015). It is clear that a better understanding of virulence on a general level could help open up avenues for future drug development approaches.

2.2.1 Acquiring access to an infection site is a first step

Looking back at Falkow's list, it is important to notice that many commensals do many if not all of these. Some cases of pathogenic bacteria can also be readily considered commensal, such as intestinal *H. pylori*, which evidence suggests may both be a protective and lesion/cancer inducing intestinal microbe. It is also known that pathogenicity capable *E. coli* exist in the intestine without causing harm. Sometimes pathogens, however, enter their symptomatic site and pathogenic state.

Margolis aims to emphasize that bacterial illnesses often result from an exaggerated immune response attempting to prevent pathogens from inhabiting unwanted areas. For example, programmed cell death and shedding of cells under infection from the urethra or intestine can be considered reasonable protective strategies against pathogenic *E. coli* in a world without medicine. An alternative approach to treating infections thus involves mitigating the immune system's excessive reaction to reduce morbidity and prevent mortality (host directed therapy). Additionally, since much of the severity of infections stems from interactions between the host and pathogen, a similar strategy can be applied to thwarting bacterial virulence by disrupting molecular processes between the host and the cell. Margolis illustrates this concept with a figure depicting pathogen-host interactions (Figure 1). In this process, pathogens initially enter a specific site (depicted by the blue box), where they replicate and establish a population without causing noticeable symptoms. Virulence occurs when they migrate to a

second site (depicted by the red box), where the presence of bacteria or their byproducts may or may not induce symptoms. For some pathogens this site is an entirely distinct physical locus, such as the urethra, but it can also be abnormal loci in the intestine such as within the epithelial cell or adhered to its surface. There may also be molecular and physiological differences between hosts such that certain individuals are more susceptible or entirely immune. Since bacterial adhesion or invasion to/of cells seems to be histologically specific, knowing these mechanisms could allow to design blockers of entrance into the symptomatic site or disrupt the pathological process in the niche after it is achieved. Such a prevention of entry to the symptomatic site can be considered one generalized form of antivirulence approaches.

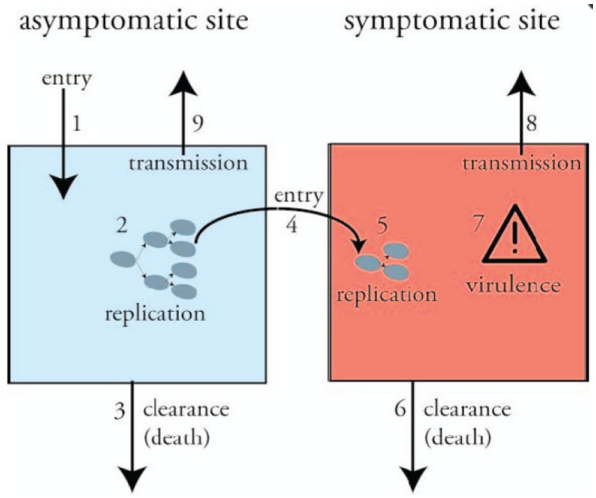


Figure 1. Enteropathogens live, replicate and are transmitted largely in the environment, but acquire genes that afford niche construction in symptomatic sites, such as within enterocytes or adhered to the plasma membrane. Image from (Falkow and Margolis 2009).

2.2 Pathogenic *E. coli* use mobile adhesin genes to adhere and achieve access to the symptomatic site

It can be said that adhesion is at the heart of most bacterial virulence and it is therefore a widely studied field of antivirulence (reviewed in Pecoraro et al. 2023). It can be seen as a manner in which the pathogen enters the space where it can cause virulence in Margolis' scheme (Figure 1). In order for the pathogen to be able to infect a cell, it needs to come in contact with it in a more permanent manner. In many loci that pathogens infect there are physical forces preventing their stay in contact with the host cell. For example, shear caused by the contents of the intestine or urinary tracts actively move pathogenic *E. coli* away from close proximity to the cells there. In addition to this, occupying a specific locus, vacuole or generating a microcolony via adhesion, can physically protect a subsection of the colony members from immune reactions of the host, such as being phagocytosed by macrophages, xenophagy, or the toxic effects of innate immune reactions.

Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize

sites that *E. coli* does not normally inhabit, such as the small intestine, brain, and the urethra (Kaper, Nataro, and Mobley 2004). Most of these reactions are mediated by distinct morphological structures that most bacteria express on their surface such as pili (also called fimbriae), and these typically are constructed of multiple repeated stalk units for building length and then a distal adherence domain, that binds to host cell surface moieties (Hacker 1992). Fimbriae are rod-like structures of 5-10 nm diameter – that are distinct from flagella, although these share structural properties. Fibrillae are similar smaller devices 2-4 nm in diameter, and are either long and wiry or curly. For example, UPEC uses type 1 fimbriae to adhere to urothelium, and these are structures of multiple subunits of fimA followed by an adhesin subunit fimH, that binds to host cell β1 and α3 integrins (Stærk et al. 2016). Similarly EPEC expresses the bundle forming pilus (BFP) from the pEAF (EPEC Adherence factor, also known as pMAR) plasmid which is made up of multiple repeating bfpA units (Giron, Ho, and Schoolnik 1991). In addition to these fimbrial adhesins, pathogens typically also have so-called single molecule (or non-fimbrial) adhesins that can be, for example, autotransporter proteins (such as intimin). These have the structure where a binding domain is transported from the periplasm to the cell-external area through a pore (discussed more in section 2.5.2). As we can notice, the genes involved in the adherence of EPEC are for the most part expressed from mobile genetic elements (plasmids and pathogenicity islands).

Pathogenicity islands can be recognized by the fact that they contain genetic content (e.g. G/C content, codon usage) that differs from the rest of the bacterial chromosome (Soares et al. 2012). Virulence genes tend to be clustered into such islands and not spread randomly in the chromosomes of pathogens. Many times these sequences are also near integrases or transposases and in prophages. This suggests that bacterial virulence is many times a result of specific acquired affordances that open the way to pathogenic lifestyles, and it is the idea in antivirulence and pathoblocker strategies to drug development to target these and design interventions that inhibit these without traditional antibiotic mechanisms of action. Since these virulence related pathways interact with many molecular mechanisms to sense outside events, there may be multiple sites where intervention can be attempted. For example, quorum sensing has been described and known for decades (De Kievit and Iglewski 2000; Mukherjee and Bassler 2019). Quorum sensing is the capacity of bacteria to recognize the presence and number of their cohorts in colonies and to initiate certain metabolic events, such as the synthesis of materials to provide biofilms, or the expression of cell surface molecules for interacting with the host (De Kievit and Iglewski 2000). In addition, many so-called two component systems have been described, which are essentially sensor-based transcription switches that pathogens use to recognize when they are in a locus suitable for expression of virulence molecules. The expression of genes from the main pathogenicity island of EPEC is regulated by both two component systems and quorum sensing related mechanisms (Sperandio et al. 1999).

The mosaic acquisition of new genetic material is a very important way of life for bacteria. An analysis of *E. coli* phylogeny based on whole genome sequences conducted by Lo et al. suggests that uropathogenic *E. coli* (ExPEC) likely evolved from commensal *E. coli* strains that initially lacked virulence factors (Lo et al. 2015). The intestines serve as the primary colonization site for *E. coli*, and the authors propose that these bacteria became pathogenic through horizontal gene transfers facilitated by processes such as transduction, transformation, and conjugation when in contact with other species within the intestine. This involved the transfer of phages, plasmids, and genomic islands from other strains to commensal intestinal bacteria. Furthermore, it's noteworthy that many uropathogenic *E. coli* (UPEC) strains, despite acquiring genetic material that enables them to cause urinary tract

infections, still exhibit commensal behavior in the intestine (Mann et al. 2017). The acquisition of genetic islands can also account for the divergence observed among different pathogens species. For instance, although *Salmonella enterica* is considered a separate species, it shares approximately 70% of its genome with *E. coli* (Karberg, Olsen, and Davis 2011). Additionally, some variants of *E. coli* are genetically closer to Shigella than to other *E. col*i strains. It's believed that the divergence between *Salmonella* and *E. coli* is a relatively recent event, primarily driven by the acquisition of pathogenicity islands (Nieto et al. 2016). Many different kinds of combinations of virulence profiles for *E. coli* exist in the wild and they are constantly evolving.

From a drug development perspective, interfering with these kinds of mechanisms is somewhat more challenging as most of these reactions will be interactions between macromolecules, for example, protein-carbohydrate or protein-protein interactions. In contrast, to small molecule-protein interactions, already existing cavities for binding or known small molecule ligands do not exist. A large part of traditional pharmaceutical development has not focused on such macromolecular interactions, but rather on small molecule ligand and protein interactions. In addition, any universal and general properties of the host-pathogen interactome are not well known due to lacking data on the topic (Crua Asensio et al. 2017). Are some PPI nodes more central and what level of redundancy exists? There is evidence that in many adhesin-ligand interactions forces are activated by tensile force and can be a magnitude of order stronger than classical non-covalent biological bonds (Dufrêne and Viljoen 2020). The *Yersinia* adhesin invasin directly engages the integrin β1 subunit with a 100-fold higher affinity than the natural ligands fibronectin, collagen, or laminin (Hamburger et al. 1999). Also, UPEC adhesin FimH is known to even increase binding upon shear using a latching mechanism (Yakovenko et al. 2008). These kind of adhesins offer various advantages to the pathogens, one of these is the positioning the pathogen close to the host plasma membrane, allowing the translocation of effector molecules via the T3SS, and another is that many of these cell molecules are central components of the host, so that evasion from pathogen binding, e.g., by mutation of adhesin binding sites, would not be compatible with their physiological role (Mix et al. 2021). In addition, the plasma membrane cell adhesion molecules are connected to the intracellular actin cytoskeleton affording bound pathogens with a means to initiate cytoskeletal organization, which is a central part of e.g. cellular invasion and niche construction by manipulating the membrane (e.g. pedestals)(Stradal and Schelhaas 2018).

2.3 The challenges of drug development against adhesins and large macromolecular interfaces

Some of the above mentioned facts obviously make adhesion a difficult target for drug development. It does not help that there are strong binding affinities and large redundancy in these interactions. *Prima facie*, there appears to be many reasons why adhesin interactions, such as that between intimin and Tir, might be considered compelling pharmaceutical targets for mitigating virulence. Firstly, Tir-intimin interaction is seemingly indispensable for the formation of actin pedestals, as evidenced in cell culture models (Cleary 2004). These actin pedestals may play a role in EPEC induced pathogenesis. Importantly also, the extracellular domains of intimin and Tir have been subjected to X-ray crystallography, enabling the creation of three-dimensional representations for *in silico* investigations (Y. Luo et al. 2000). And furthermore, a notable advantage is that most adhesin targets are extracellular. This

bypasses most of the conventional pharmacokinetic challenges associated with drug development, as there is no need to design compounds capable of accessing intracellular sites or ensuring oral bioavailability with sufficiently high plasma concentrations etc. And as the interaction occurs outside of the bacterium, this target circumvents one of the most formidable obstacles in antimicrobial drug development – penetration of the membranes and the issue of efflux pumps. However, it is imperative to acknowledge that the published NMR and x-ray crystal structures reveal some problematic features of the structure. One of them is that the (1,335 $Å^2$) buried Tir-intimin interface "is generally flat and uncharged" (Y. Luo et al. 2000). In addition, as we will see later, the topology of the binding interface is largely defined by tertiary structure, which significantly increases the challenge. It is notable that several industrial partnerships have been initiated in the last decades to discover antimicrobial PPI's, with little success yet, suggesting that this may be more difficult than what one would think.

For many decades protein-protein interactions were considered intractable by small molecule therapy. Although today there are existing protein-protein inhibitors, this way of thinking still has some merit to it (Lu et al. 2020). Most of the traditional drug target proteins interacting with small molecules have specifically evolved to solve the problem of binding to a small molecule with multiple non-covalent interactions. They have evolved to solve this same recognition problem with respect to their natural small-molecule substrate or ligand; even though the observed binding of the natural ligand is often relatively weak, the protein almost always must develop substantial 'intrinsic binding energy' with the ligand to perform its function. The canonical examples of this are enzymes and monoamine receptors that solve the problem with topology and contain a preformed cavity or clefts for binding their natural ligand, which allows multiple binding interactions to be focused upon the small molecular volume the ligand presents while protecting the molecule from solvent interactions, which generally would counter binding interaction energies.

A large proportion of the traditional drug development methodology, including high-throughput screening, virtual screening and docking methodologies were developed to identify optimal binding sites and predict binding modes of compound collections in such targets from x-ray crystallography experiments. For example, the original data set (Cheng et al. 2007a) used to develop and validate the SiteMap algorithm and other druggability tools contained 538 targets, but only one of these represents a protein-protein interface – MDM2/p53 (Halgren 2007). SiteMap is a method used to predict locations on three dimensional representations of proteins for "druggability", that is, whether or not they are likely to be good binding sites for small molecule drugs. Therefore it is not really known how well this (or other druggability) methodology generalizes to flat PPI's, despite it having been highly successful (86%) in classifying the previously mentioned validation data set.

The discovery of energetic "hotspots" in protein-protein interactions by Clackson and Wells. (1995) changed the mindset on the tractability of PPI's. They demonstrated that, while the binding interface between proteins is larger than traditional drug pockets, the majority of the binding energy comes from contacts with only a small number of (typically 3) amino acid residues on each protein using alanine scanning mutagenesis (Clackson and Wells 1995). This raised the prospect of inhibiting particular protein-protein interactions without needing to block the entire interface. This observation has been confirmed by analysis of various PPI interfaces, but it remains unknown in detail and to what extent such hotspots are druggable. In the last few decades several dozen protein-protein interaction inhibitors have been discovered and some brought to clinical trials, but this is not enough to draw systematic conclusions from. For example, Alzyoud et al. analyzed 330 known PPI-interfaces (in 12

targets) using SiteMap in order to get an idea on how well it works on such non-traditional targets (Alzyoud et al. 2022). Many of these sites receive fairly low scores, and for ZipA, not a single druggable site was recognized. ZipA is a membrane bound cell division protein of *Eschirichia coli* which interacts with FtsZ to recruit cell division proteins to the septum, and therefore could be a good antibiotic target. A few inhibitors of ZipA-FtsZ have been described in the literature; however, these do not bind to a cavity in the PPI interface, but rather to high energy hotspots elsewhere on the protein, which the authors suggest explains why efforts over the last 20 years have failed to generate a ZipA inhibitor that can reach clinical trials (Alzyoud et al. 2022). Obviously, since druggability metrics and docking methodologies are at the end of the day based on physical forces between atoms, one can expect these to work *to some degree* independent of the molecules used.

2.3.1 Techniques and methodology for discovery of PPI inhibitors

Protein-protein interaction (PPI) interfaces exhibit considerable variability in their characteristics. Some interfaces consist of a single contiguous segment of residues, while others are composed of multiple non-contiguous segments of amino acids. Furthermore, the epitope, or binding site in the hotspot region, of a PPI interface can be associated with various levels of structural organization. It may constitute a primary structural element, a secondary structure such as a helix, or even a tertiary globular structure. Generally, it is believed that druggability, or the feasibility of designing small molecule inhibitors, tends to decrease in that order. Primary epitopes can be inhibited by small linear peptides even as these display no higher order structure(Lee et al. 2013). Secondary epitopes display certain regular interfaces such as alpha helices that have recurring structural properties allowing for rational design of mimetics(Pelay‐Gimeno et al. 2015), whereas tertiary shapes vary much more and the interfaces are dynamic (Arkin, Tang, and Wells 2014). In certain instances, successful inhibition of tertiary epitopes with binding affinities in the nanomolar (nM) range has been achieved, though ligand efficiencies for such cases are generally moderate. An example of this is the development of a macrocycle against IL-17 by Ensemble Therapeutics (Zhang and Dömling 2022).

A quick look at the literature suggests that there are a few approaches that seem to be used particularly frequently in targeting PPI's. One approach is the combination of binding assays and biophysical methods, such as x-ray crystallography to screening (Magee 2015). A popular approach is also so-called structure activity relationship by NMR (Shuker et al. 1996) and derivatives (Larda et al. 2023) where a biophysics-based structure activity relationship (SAR) cycle is used. These approaches use the knowledge acquired from e.g. fragment compound binding to the protein of interest for the rational design of a large ligand e.g. The basic idea is to design novel ligands based on the true empirical position and spatial orientation data obtained by e.g. linking multiple fragments in the design of a larger drug molecule. This appears to work particularly well for PPI's because the hotspot residues are typically not in one contiguous segment and there is typically no known native small molecule binder. Thus creating the empirical knowledge about real world binding is helpful to achieve a form of "ground truth" for computational modeling.

Another theme in the literature is the development of specialized chemical screening libraries that are enriched for PPI inhibitors (Milhas et al. 2016; Ran and Gestwicki 2018). Essentially, these collections tend to be more "natural product-like", having higher average molecular mass and more stereocenters (Silvian, Enyedy, and Kumaravel 2013). For example, Vincendeau et al. screened a natural product library to identify an anthraquinone derivative

that inhibits binding of NEMO-Ubiquitin, a large interface area of 4520 Å^2 (Vincendeau et al. 2016). Similarly, inhibitors inspired by natural products also led to the discovery of the first non-azaphilone containing chlorofusin as an inhibitor of MDM2/p53 (Cominetti et al. 2015). Furthermore, throughout history, natural products have been described that can bind SH2-domains (Sperl, Seifert, and Berg 2009), microtubules, such as taxanes and vinca alkaloids (Stanton et al. 2011), and certain transcription factors (Hollis et al. 2012) and other protein-protein interfaces, suggesting that such interaction may occur more frequently than currently known in biology at large.

With respect to purely computational approaches, in order to address the issue that traditional computational drug discovery tools are not suitable for PPI's, some authors have attempted to create more complex approaches e.g. by combining various pharmacophore models and then using consensus scoring (Geppert et al. 2012). This appears to decrease the amount of false hits proposed by either method alone.

2.4 Enteropathogenic *Escherichia coli*

2.4.1 Demonstrating the pathogenicity of Enteropathogenic *Escherichia coli*

The experimental of this work evolves around enteropathogenic *Escherichia coli* (EPEC), which today is considered one of the many *E. coli* pathovars. It is believed to cause intestinal infections, sometimes chronic, with symptoms of diarrhea and malabsorption of nutrients and to be a large cause of infant mortality in the developing world (Theresa J. Ochoa and Contreras 2011). The discovery of a significant portion of bacteria responsible for infectious diseases occurred during the "golden period of bacteriology" in the 1870s and 1880s. But EPEC and other pathogenic strains of *E. coli* were not initially recognized as disease-causing pathogens in the early days of modern medicine. The used experimental methods, such as colony screening on agar plates, did not allow to easily isolate and differentiate the causal agent (virulence causing gene clusters) from the healthy colonies as commensal *E. coli* are the predominant facultative anaerobe of the human gut microbiota. So, as early as 1885, Theodor Escherich described *Escherichia coli* without attributing any pathogenic properties to it, ironically, given that he was interested in identifying the source of "summer diarrhea," a condition that was later commonly attributed to pathogenic *E. coli* serovars, particularly EPEC. Summer diarrhea was a recurrent enteric infection prevalent during the summer months of each year and was particularly lethal among newborns. Nonetheless, as early as 1898, serum from diarrhea patients agglutinated strains of *E. coli* recovered from other patients in the same outbreak but not those isolated from a control patient, suggesting that some *E. coli* might be enteropathogens, not to mention that the infections seemed to follow epidemic patterns (Robins-Browne 1987).

The eventual discovery of EPEC as an intestinal pathogen is often attributed to Beaven and Bray in the 1940's (Bray 1945; Sine nomine 1973). By then, summer diarrhea had mostly vanished from the developed world, although why this happened is not really known and is still being debated (Nataro and Kaper 1998; Anderson, Rees, and Wang 2020). However, severe hospital outbreaks continued to occur on a rare basis during the 1940s and 1950s, which motivated research on the topic. During this time, Beaven and Bray studied infantile diarrhea and subsequently claimed that an *E. coli* strain is the cause of summer diarrhea (Bray 1945). Interestingly, one of the properties used to recognize the pathogen was the smell, as

the cultures of the pathogen smelled similar to the patients, leading the researchers to suspect that the *E coli* strain could be the causative agent of the disease. They isolated this strain from a patient with summer diarrhea, raised antibodies against it, and by this method they were able to demonstrate the first *E. coli* pathotype (Sine nomine 1973).

In the years following the initial discovery of pathogenic *E. coli*, several additional pathogenic serovars were identified and Fritz Kauffman (1944) introduced a systematic serotyping approach for *E. coli*. Today it is generally accepted that there are at least six distinct intestinal *E. coli* pathovars with additional pathovars external to the intestine, infecting urinary tracts and brain for example (see Table 2) (Kaper, Nataro, and Mobley 2004). These classificatory systems are far from perfect because 1) antigens on the bacteria do not determine virulence capacity, and 2) clones displaying properties making them difficult to classify, for example hybrid EPEC/UPEC exist (Nascimento et al. 2021). However, it is likely that these pathotypes have become prevalent in a continuous mixture of genetic material during evolution (Kaper, Nataro, and Mobley 2004) and represent more stable clones.

Most significantly, it was demonstrated by experimental human challenge experiments in the 1950s that many *E. coli* strains cause diarrhea (Neter and Shumway 1950; Ferguson and June 1952; June, Ferguson, and Worfel 1953). In these experiments, cultures obtained from infantile gastroenteritis were administered to adult volunteers via oral ingestion. The test subjects frequently exhibited symptoms in a dose-dependent manner when exposed to these isolates. Conversely, *E. coli* cultures from healthy newborns did not elicit similar responses, providing robust and high quality support for the view that these bacteria are indeed pathogenic *E. coli* strains. Despite this compelling evidence, the primary technological method for distinguishing pathogenic from non-pathogenic *E. coli* at the time was serotyping and when ST toxin containing *E. coli* were discovered in the 1970's some, some researchers doubted that EPEC strains may only be strains that have lost their capacity to produce this toxin over many years of laboratory culture, so Levine *et al*. repeated such studies with EPEC 2348/69 showing that it specifically is a human pathogen despite it lacking production of toxins (Levine et al. 1985). After this, this particular strain has become the most widely used laboratory model of EPEC pathogenesis, although today it is known that there are other clones that do display minor variations in the virulence pathways. Indeed, in the original publication, Levine *et al*. also studied other EPEC's that are non adherent to cell culture, which nevertheless caused human disease. Today it is known that so called atypical EPEC, variants that are missing a virulence plasmid are common worldwide (Scaletsky et al. 2010; Rocha et al. 2011; Kralicek et al. 2022). These strains are capable of lesioning the intestines and diarrheal disease, although significantly less virulent. It is possible that there are as of yet uncharacterized clones that display even lower virulence.

Table 2. A list of the currently recognized main *E. coli* pathotypes according to Kaper Nataro and Mobley (2004).

Intestinal	**Extraintestinal**
enteropathogenic *E. coli* (EPEC)	uropathogenic (UPEC)
enterohaemorrhagic *E. coli* (EHEC)	meningitis-associated (NMEC)
enterotoxigenic *E. coli* (ETEC)	

enteroaggregative *E. coli* (EAEC),	
enteroinvasive E. coli (EIEC)	
diffusely adherent E. coli (DAEC)	

2.4.2 EPEC and attaching and effacing lesions

The study of bacterial pathogenicity on the molecular level really picked up from the 1980's onward. With respect to EPEC, Cravioto et al. discovered a remarkable fact, that most EPEC strains adhere to (carcinoma) cell culture monolayers, whereas non-pathogenic *E. coli* do not (Cravioto et al. 1979). This was the first phenotypic determinant in addition to serotyping that could distinguish EPEC from non-pathogens. In the study of pathogens, such a model is very important as pathogens are typically human limited and studying them using murine or insect models is difficult or impossible. EPEC have been administered to rodents, but do not display normal pathology in this environment (Mundy et al. 2005) although mice have their own analogous pathogen, *Citrobacter rodentium* often used as a surrogate for EPEC in research. Humans are considered to be the main reservoir of EPEC, even though there are reports of EPEC in many different domestic and wild animals (McDougall et al. 2023). The utilization of this *in vitro* human cell culture model played a pivotal role in enabling future mutant and imaging studies. Notably, the publication of electron microscopy images of EPEC infections led to an intriguing observation. In the cultured human carcinoma cell lines, electron-dense spots were found to colocalize with the bacteria (Ulshen and Rollo 1980; Moon et al. 1983). Examination of infected epithelial cells revealed that EPEC induced profound cytoskeletal alterations, pseudopod-like protrusions of up to 10 µm in size. Pseudopods are temporary arm-like projections of eukaryotic cell membranes that emerge in the direction of movement. Remarkably, the bacteria situated themselves "on top" of these protrusions, leading to the coining of the term "pedestals" to describe this unique phenomenon (Figure 2).

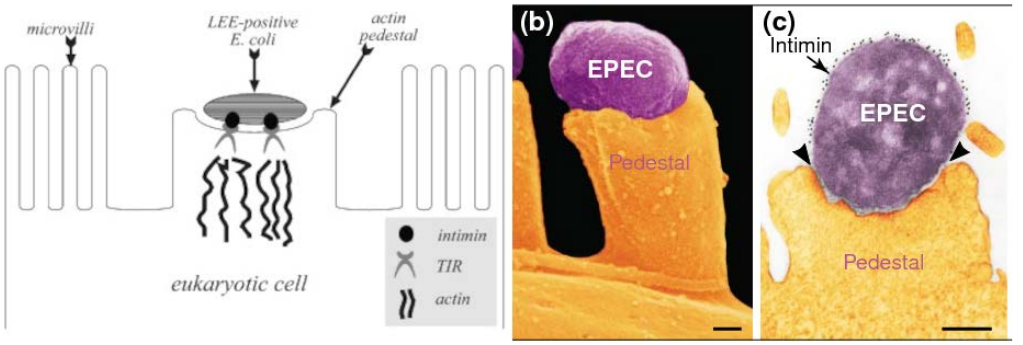


Figure 2. A) A schematic of actin pedestal and effacement of microvilli. EPEC infection causes the effacement of normal microvilli, which is important for the normal function of the intestines. When EPEC hijacks the cellular actin nucleation machinery, it creates an abnormal actin structure called a "pedestal". From Jores et al. B) pseudo colored electron microscopy images of EPEC induced actin pedestals. C) Intimin is displayed on the outer membrane of EPEC

As it was known that actin is the major component of cytoskeleton and in microvilli in particular, Knutton et al. (1987) proposed that the electron dense material in the images could be high in actin. In actin staining experiments, using fluorescein-labeled phalloidin, their work showed that both EPEC infected *ex vivo* transplants and cultures of Caco-2 cells displayed intense fluorescent spots, and that other adherent bacteria did not cause these. These abnormal actin polymerization events were postulated to explain why attaching and effacing (A/E) pathogens induce lesions and the effacement of microvilli previously seen in biopsies, since actin is a very central component in the structure of microvilli, even though the simple cell culture model does not display microvilli. Remodeling the actin cytoskeleton is now known to be a central method for many pathogens to enter cells or move around them (Stradal and Schelhaas 2018).

The invention of this assay – used also in the studies of this dissertation – was a major breakthrough in that it allowed for cost-effective high-throughput *in vitro* study of isolates and mutants. Previous to this, the only way to study the pathogens was serotyping, adherence and electron microscopy. It also suggested that the lesions were not simply passively destroyed epithelium (e.g. by a toxin), but that the lesions involved active cellular mechanisms (e.g. actin nucleation machinery) hijacked by the pathogen. Subsequently it was shown via immunofluorescence microscopy that many other cytoskeletal proteins and actin nucleators are present in EPEC-induced actin pedestals. For example, alpha-actinin, talin, erzin, myosin-light chain, VASP WASP and the Arp2/3 complex were all demonstrated (Finlay et al 1991; Finlay et al. 1992; Kalman et al. 1999). The following genetic screens, using the FAS assay and random mutagenesis, also led to the identification of the first essential virulence factor for EPEC virulence and attachment to cultured cells: an outer membrane adhesin, intimin, encoded by the *eae* gene (Jerse et al. 1990; Jerse and Kaper 1991). This led to the theory that the adherence of EPEC to host cells using these adhesins was a central first step to the following pathogenic cascades.

2.4.3 The four stage model of EPEC adhesion and virulence

Most if not all bacterial infections initiate with the pathogen adhering to the host cell in one manner or another (Pizarro-Cerdá and Cossart 2006). After achieving such proximity it is possible for the small sized bacterial organism to produce compounds in high enough localized concentrations to cause virulent physiological processes. Many virulence molecules are targeted to intracellular organelle membranes in order to achieve bioactivity with picomolar concentrations (Schlumberger et al. 2005), due to the localized nature of the assembly of compounds.

In the scenario of EPEC, a widely acknowledged model delineates three sequential stages of pathogenesis, as illustrated in Figure 3 (Mare et al. 2021). Initially, external adherence is facilitated through peripheral appendages, such as pili, including the *E. coli* common pilus and notably the bundle-forming pilus (BFP) of EPEC, which is encoded by a plasmid (pEAF) comprising 14 genes (Brinkley et al. 2006). These structures contribute to the formation of microcolonies and an adherence pattern termed "localized adherence" (Vuopio-Varkila and Schoolnik 1991). However, certain strains classified as atypical EPEC naturally lack this plasmid, resulting in diminished virulence and absence of localized adherence in microcolonies, notwithstanding their capability to induce attaching and effacing (A/E) lesions (Scaletsky et al. 2010; Rocha et al. 2011). Nevertheless, the *E. coli* common pili also play a pivotal role in the initial adherence phase (Saldaña et al. 2009). Interventions aimed at blocking these pili could potentially impede cell adherence, thereby mitigating virulence,

without necessitating bacterial eradication. Here we can also appreciate the difficulty of designing adhesion blockers, as it is common that there is a lot of functional redundancy (Krachler and Orth 2013).

Subsequently, during the second phase, the type three secretion system needle facilitates the injection of virulence-related molecules into the host cell (Pinaud, Sansonetti, and Phalipon 2018). The T3SS is a large needle like device (Figure 5), created from distance-adding repeating bulk subunit, espA, and distal pore-forming subunits espB and espD (Kenny et al. 1996; Cleary 2004). These form a conduit from the periplasm to the host cell cytoplasm, through which multiple virulence molecules are injected into the host. In this process, the translocated intimin receptor (Tir) is also delivered into the host cell cytosol and subsequently migrates to the plasma membrane, where it serves as a docking site for EPEC through interaction with its ligand molecule, intimin (Hartland et al. 1999). This results in an intimate attachment of the bacteria with the host, characterized by close and robust adherence of the pathogens to the plasma membrane (see Figure 3).

Once intimate attachment occurs, host cellular processes initiate the development of pathological changes, including the formation of attaching and effacing lesions (Gad Frankel and Phillips 2008; Kalman et al. 1999). These lesions are characterized by effacement of the intestinal epithelial microvilli and abnormal condensation of actin filaments into the pedestal. A very similar pattern is shared by many other pathogens, that use for example a trimeric autotransporter adhesin to acquire intimate attachment and then inject effectors using a T3SS (Kiessling, Malik, and Goldman 2020).

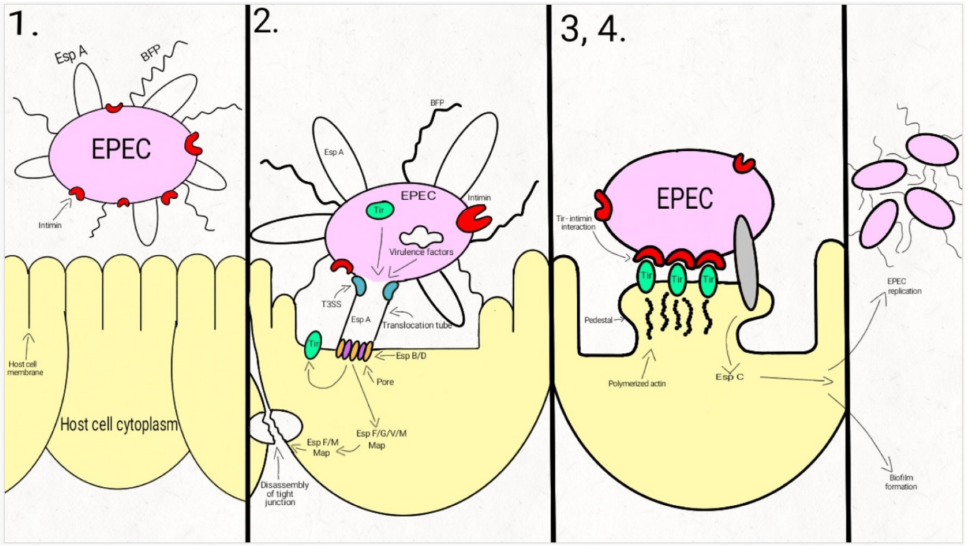


Figure 3. The four step model of EPEC infection, where EPEC 1) uses external appendices to locate and bind the enterocyte, then 2) uses the T3SS needle to inject Tir and other virulence molecules into the host. After this 3) abnormal actin polymerization occurs and EPEC becomes intimately attached to the membrane. 4) After colonization, further growth of new members to the colony occurs and biofilm materials are generated to protect the colony. From Mare et al. 2021.

2.5 Molecular mechanisms of EPEC A/E lesions

2.5.1 The injection apparatus and virulence inducing effectors of EPEC

Many pathogens cause their effects by producing specific virulence factors, proteins that enable them to exert their harmful effects on the host cell (Leitão 2020; Diard and Hardt 2017). These factors often originate from prophages or other mobile genetic elements, although the exact reasons for this phenomenon remain unclear (Rankin, Rocha, and Brown 2011; Keen 2012). Genes associated with virulence are typically found clustered together in genomic islands near insertion sites rather than randomly dispersed throughout the genetic material (Kaper and Hacker 1999). When these genomic islands carrying virulence genes were first identified as agents responsible for causing disease in UPEC by Jörg Hacker and colleagues in 1994, they were termed 'pathogenicity islands' (Blum et al. 1994). Pathogenicity islands have since been observed not only in other pathogenic *E. coli* strains but in most pathogenic bacteria.

The capacity for EPEC to cause pathogenic outcomes, in contrast to commensal *E. coli*, stems from its acquired virulence genes located on a pathogenicity island. McDaniel et al. were the first to identify the presence of this pathogenicity island in attaching and effacing (A/E) pathogens, naming it the Locus of Enterocyte Effacement (LEE) (T. K. McDaniel et al. 1995). This genetic island is a 35 kilobase prophage acquired through horizontal gene transfer and encompasses multiple operons encoding many of the proteins essential for the virulence of EPEC and other A/E pathogens (Figure 4). Although certain individual effector proteins may be encoded outside of the island, the transfer of the LEE to nonpathogenic *E. coli* laboratory strains enables them to induce actin pedestals (Timothy K. McDaniel and Kaper 1997; Elliott et al. 2000).

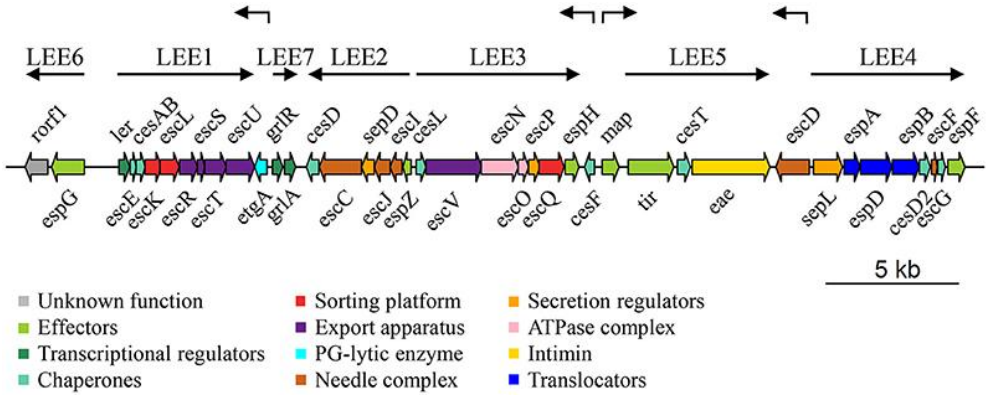


Figure 4. The structure and contents of the Locus of enterocyte effacement pathogenicity island. The genes code for the T3SS, chaperones and effectors. It also encodes its own regulators. From Gaytán et al. 2016.

The LEE contains genes responsible for coding the components of the type three secretion system (T3SS), including the needle components themselves, the required translocators and chaperones, the effectors that are injected into the host cell, and the autotranspoerted cognate ligand of Tir, intimin. Among the injected effectors are espF, espG, espH and Tir. Type three secretion systems are structures resembling needle-like molecular syringes, which Gram-negative pathogens use to deliver bioactive molecules into host cells (Figure 5). Recent

Cryo-eM studies have revealed the structure of EPEC T3SS in remarkable detail (Lyons et al. 2021; Zheng et al. 2021). At the base of this needle apparatus, there's a port where chaperones guide the effectors called the sorting platform. Then, an ATP-hydrolyzing motor drives the effectors through a narrow shaft, around 2-4 nanometers wide. This passage traverses both the bacterial outer membrane and the mammalian plasma membrane to finally reach the host cell cytoplasm.

Upon interaction with enterocytes, EspB and EspD proteins are integrated into the host plasma membrane, assembling to form a translocation pore (Ide et al. 2001; W. Luo and Donnenberg 2011), while long shafts composed of EspA filaments extend from the needle apparatus (see Figure 5). These components of the T3SS needle complex are commonly referred to as "translocators".

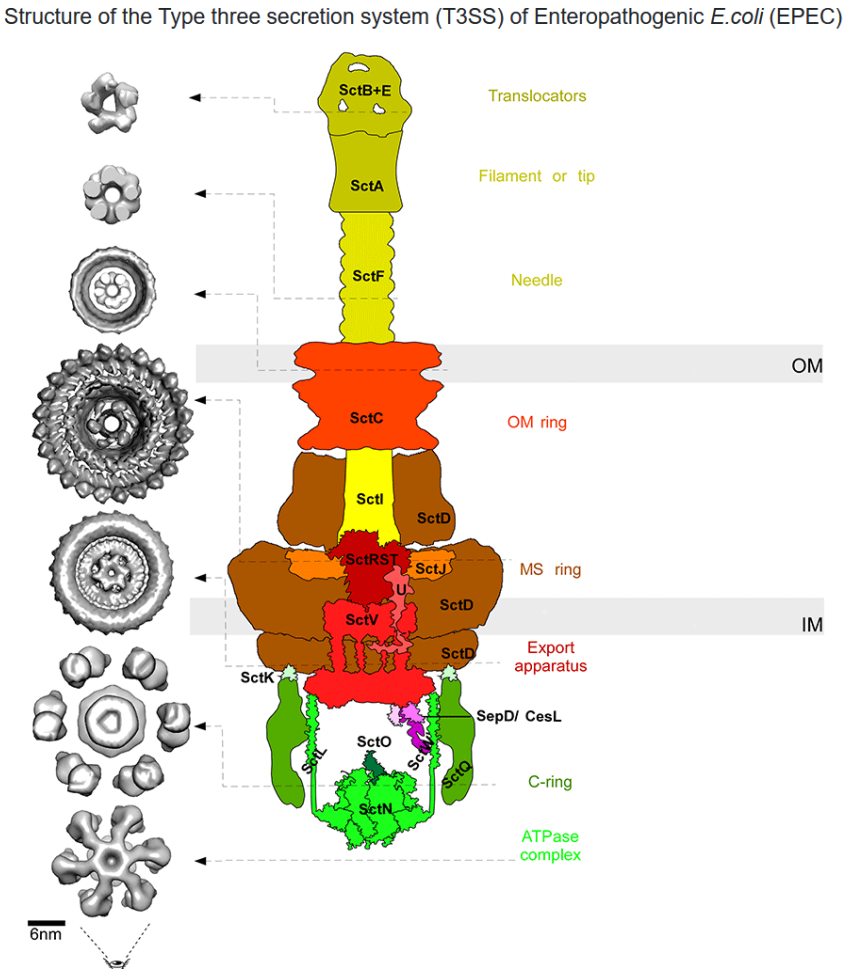


Figure 5. The general structure of the EPEC T3SS needle that is encoded by the LEE pathogenicity island. A needle complex is formed on the bacterial membrane, which has a motor to pump the proteins and chaperons required through the needle and filament. At the other end of the filament are pore forming translocator proteins, which open a door for virulence molecules, such as the translocated intimin receptor to enter the host cell. On the bacterial side there is the needle complex which involves a atp-consuming motor and a port where

chaperones bring effectors to be injected. Created with STEPdb. The names shown here are the common nomenclature names and their corresponding partners in EPEC are provided in the legend.

Table 3 Legend for Figure 5 describing the roles and names of the individual subunits that construct the type three secretion needle in EPEC

Export apparatus or Translocase	Minor component, external to channel	SctU	EscU
Export apparatus or Translocase	Translocase channel	SctR	EscR
Gatekeeper	Subunit 1 of SctW chaperone heterodimer	-	SepD
OM ring	Secretin	SctC	EscC
Translocators	Translocon pore protein	SctE	EspD
ATPase complex	Hexameric ring-structure ATPase	SctN	EscN
Needle	Needle component	SctF	EscF
Cytoplasmic ring	Connector of Pods with SctD	SctK	EscK
Gatekeeper	Subunit 2 of SctW chaperone heterodimer	-	CesL
ATPase complex	External stator connecting ATPase and Cytoplasmic ring	SctL	EscL
Export apparatus or Translocase	Translocase channel	SctT	EscT
Export apparatus or Translocase	Translocase channel	SctS	EscS
IM ring	Major IM ring component	SctD	EscD
Translocators	Translocon pore protein	SctB	EspB
OM-IM connector	Inner rod	SctI	EscI
Gatekeeper	Gatekeeper/Affinity switch	SctW	SepL
Translocators	Tip/filament protein	SctA	EspA
Cytoplasmic ring	Component of 6 Pod assembly	SctQ	SepQ
ATPase complex	Central stalk, inserting in ATPase ring	SctO	EscO
IM ring	Lipoprotein ring component	SctJ	EscJ
Export apparatus or Translocase	Major component, external to channel	SctV	EscV

This shaft has a lumen through which unfolded effector proteins travel into the cytosol where they then are assembled and folded into their functional forms. Chaperones are involved in this and they are named chaperones of *E. coli* secreted proteins (Ces), so for example CesT is

the chaperone necessary for translocating Tir, and the deletion of this leads to the accumulation of Tir in the bacterium and the incapacity to efficiently translocate. CesT is able to bind to and mediate the translocation of several other effectors, even non-LEE effectors(Little and Coombes 2018; MacDonald et al. 2017). There are typically n-terminal motives of amino acids, called the chaperon binding domain (CBD) that bind the substrate, and this is the case with cesT and Tir (Little and Coombes 2018). The n-terminus, is not strictly necessary for actin polymerization, since if the receptors are transfected to cells and the n-terminus removed, the receptor can, nevertheless, be phosphorylated, dimerize and activated (Campellone et al. 2002; 2004).

The expression of LEE is under the control of multiple regulatory networks. Within the LEE1 operon, the first open reading frame encodes the Locus of Enterocyte Effacement-encoded Regulator (Ler), situated within the island (Figure 4)(Berdichevsky et al. 2005). Ler functions as a positive regulator of LEE genes by competitively interacting with its counterpart, H-NS (Winardhi et al. 2014). Conversely, H-NS acts as a suppressor of LEE gene expression by forming rigid filament structures bound to DNA. However, this process is disrupted and replaced by Ler, although the exact mechanisms remain unknown (Winardhi et al. 2014). Despite the precise mechanisms of Ler regulation not being entirely clear, it is understood that Ler binds to DNA non-cooperatively, inducing changes in its rigidity and resulting in the formation of toroidal nucleoprotein complexes along DNA.

Intriguingly, Ler also governs the expression of certain genes outside of the LEE, such as the cytotoxin *espC*, which is secreted in vesicles rather than through the T3SS (Navarro-Garcia et al. 2014). The four-step model stipulates that cytotoxins such as espC likely aid in allowing the microcolony to dissipate after the infected site is no longer needed. Moreover, the plasmid-encoded regulator (per) from the EPEC plasmid pEAF directly activates the region of the LEE1 operon encoding Ler(Mellies et al. 1999). Additionally, Integration Host Factor (IHF) serves as a direct activator of Ler and binds upstream of its promoter. IHF is a DNA-binding and -bending protein with roles in local DNA structural organization and transcriptional regulation in Gram-negative bacteria. Notably, Ler is targeted by the T3SS-inhibiting antivirulence compound aurodox, originally extracted from a *Streptomyces* strain and used as a control substance in the experimental of this work (McHugh et al. 2018).

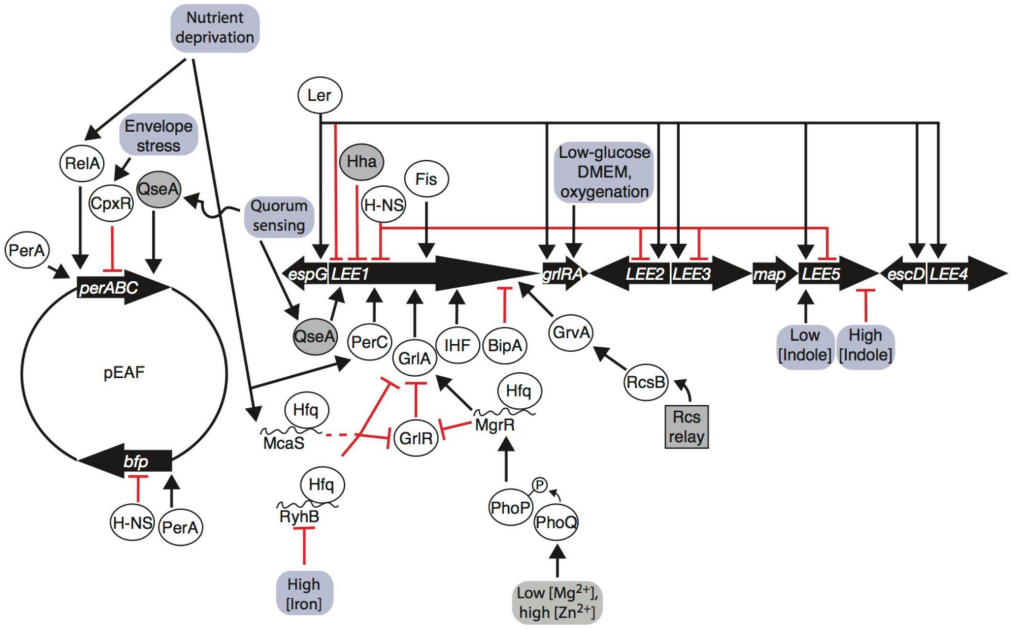


Figure 6. The regulation of the pathogenicity island, the locus of enterocyte effacement, is regulated by a complex network of switches connected to sensors of environmental cues such as various available nutrients, other bacteria for quorum, and recognizing host (Platenkamp and Mellies 2018).

A positive regulatory loop exists among Ler, GrlA, and their corresponding genes. GrlA, a regulator encoded within the LEE pathogenicity island, serves as an activator of *ler*, while Ler reciprocally activates *grlRA*, forming a feedback loop where a protein product triggers the transcription of another, which in turn activates the transcription of the original protein's gene (Figure 6)(Mellies et al. 1999). Notably, Ler's activation of *grlRA* requires the presence of H-NS, whereas GrlA's activation of *ler* is independent of this factor. Quorum sensing mechanisms also influence Ler regulation, with LuxS playing a crucial role in autoinducer molecule synthesis. The quorum-sensing *E. coli* regulator A (QseA), found in LuxS systems, promotes *ler* transcription (Russell et al. 2007). Fis, a nucleoid-associated protein vital for EPEC's formation of attaching and effacing lesions, contributes to Ler expression activation. Furthermore, BipA, a ribosomal binding GTPase and a significant regulator of EPEC virulence, exerts transcriptional control over Ler and other genes from an upstream position (Grant et al. 2003). Additionally, Ler protein engages in self-regulation by repressing its own transcript within the LEE1 operon through DNA looping, thereby impeding the progress of RNA polymerase (Bhat et al. 2014). The transcription of virulence in EPEC is affected by so many pathways, that it may well be possible that compounds could be uncovered or designed to interfere with virulence on the transcriptional level.

2.5.2 The structure and function of intimin

A non-injected virulence molecule that is encoded in the LEE pathogenicity island is the Tir ligand intimin. Intimin is a rod shaped reverse autotransporter consisting of a periplasmic lysin domain, β-barrel integrated in the outer membrane, repeated immunoglobulin-like globular domains and a distal lectin-like adhesin domain (Y. Luo et al. 2000)(Figure 7). Bacterial autotransporters are similar to classical autotransporters in that they are large polypeptides that transport a part of themselves, called a passenger, through the cell wall via a

pore that is also a part of the autotransporter protein. The term inverse refers to the fact that the passenger is the C-terminal instead of the N-terminal. The lysin domain of intimin binds to molecular peptidoglycan in experiments and is believed to help the entire structure to bind to the cell wall but additionally to aid in the dimerization of intimin (Leo et al. 2015; 2016). The authors put forward evidence suggesting that such dimerization and peptidoglycan binding are likely general features of LysM-containing inverse autotransporters (reviewed in Leo et al. 2015).

β-barrels are domains specific to Gram negative bacteria, mitochondria and chloroplasts that are involved in nutrient uptake, adhesion and protein secretion (Doyle and Bernstein 2019). Almost all of the proteins anchored to the outer membrane have a β-barrel (Doyle and Bernstein 2019), and autotransporters use this as a pore to secrete their functional cleavable or non-cleaved "passenger" domain (Clarke et al. 2022). In the case of intimin, the passenger domains are not cleaved and are a few structural ig-like globular domains and the distal adhesin domain that binds to Tir (Oberhettinger et al. 2012). Recent work also suggests that there must be two additional domains, named D0 and D00 immediately preceding the domains in PDB structure 1F02 (Weikum et al. 2020). The authors propose that this kind of domain organization would allow the passenger region to display varying rigidity and flexibility, which is necessary for bacterial adhesins under shear (Persat et al. 2015). This is similar to how boats are tied to piers or anchored to the sea bed using flexible ropes and not rigid particles, as such a structure absorbs some of the energy from the shear of the sea, while keeping the boat in place. D00 appears to be entirely rigid with the aim of protruding the adhesin from the cell wall, whereas the linker region of D00 and D0 is flexible, allowing the lectin (D4) to reach further, perhaps similar to a rigid fishing rod with a line. This is interesting because these structures could possibly allow for the development of allosteric inhibitors of intimin, if a small molecule could be uncovered that could change the conformation equilibrium, particularly since this kind of mechanism has already been demonstrated for inhibitors of tertiary protein-protein interactions.

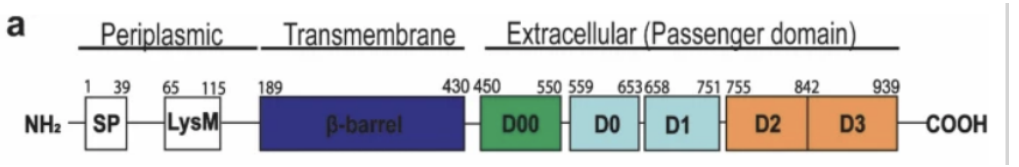


Figure 7. Domain architecture of E. coli intimin is represented based on the PDB structures exhibiting the periplasmic domain (PDB: 2MPW), including a signal peptide (SP), transmembrane β-barrel (PDB: 4E1S) and extracellular domains (PDB: 1F02). The Tir-binding site is located on domain D3. From Weikum et al 2020.

The architecture of intimin closely resembles invasin, an adhesin utilized by *Yersinia* pathogens to engage with β1-integrins. While intimin is proposed to serve a similar function, the specific target molecule it binds to remains elusive (G. Frankel et al. 1996; Hartland et al. 1999; Sinclair and O'Brien 2004). Despite the lack of sequence homology, the C-terminal domain of intimin shares topological similarities with C-type lectins (Batchelor et al. 2000). Given the role of C-type lectins in recognizing cell-surface carbohydrates, it is plausible that intimin may interact with other host membrane ligands (in addition to Tir), potentially in a concurrent manner, considering the adjacency (non-overlap) of the lectin binding site to that of the intimin binding site. This observation could account for the observed tissue tropism of various intimin subtypes as multiple immunogenetically distinct subtypes of intimin exist

across different bacteria, with EPEC 2348/69 belonging to the alpha type. The subtype influences the tissue specificity of bacterial binding within the intestine (G. Frankel et al. 1996). EPEC (intimin-α) adheres to the small intestinal mucosa, while EHEC (intimin-β) adheres to the follicle-associated epithelium of Peyer's patches in the small intestine.

Both intimin and invasin passengers show an equivalent high level structural composition and similarity to E-cadherin extracellular extension (Login et al. 2018). Cadherins are a family of calcium-dependent cell adhesion proteins that play crucial roles in cell-cell adhesion, tissue organization, and signaling during development and throughout life. They are integral membrane proteins found on the surface of cells, particularly in adherens junctions, which are specialized cell-cell junctions. Cadherins also modulate their flexibility and rigidity similar to intimin but using calcium binding in the connector regions. It has been shown that the soluble extracellular domain of E-cadherin interferes with EPEC adherence via interaction with the Tir-intimin complex *in vitro* (Login et al. 2018). The significance of this for real life infections is not clear, However, similar to β1-integrin, E-cadherin bound to intimin, although at lower levels than to the Tir-intimin complex.

2.5.3 The structure and function of the translocated intimin receptor

The translocated intimin receptor is the cognate of intimin. After the discovery of intimin, its receptor was demonstrated to be a 90 kDa protein expressed on the apical side of intestinal epithelia. Previous research had indicated that the *Yersinia* adhesin invasin – sharing significant structural similarity with intimin – binds to cell surface integrins. Thus, at the time, the receptor was named hp90 for human protein 90 kDa. However, to the surprise of many, it was soon shown by Kenny et al. via multiple clever experiments where antibodies were raised to a bacterial secreted protein but also cross-reacted with hp90, that this protein *is indeed of bacterial origin* (Kenny et al. 1997). The entire scheme of encoding a cell surface receptor with motifs of mammalian proteins was an unheard of pathogen stratagem towards host colonization. Even today, this remains unparalleled and has yet to be observed in other pathogens. Subsequently, Kenny and colleagues renamed the protein the translocated intimin receptor (Tir), the name most frequently used today. As far as I am aware, the evolutionary significance of this has never been discussed in the scientific literature. In the case of native host cell surface receptors it has been suggested that pathogens target multiple cell junction and surface moieties because it is challenging for the host to counterstrike by mutating these, as their primary function is so essential (Mix et al. 2021). However, the odds must be even worse for the host, when the receptor used by the pathogen is under the control of the pathogen's chromosome and thus essentially out of the control of the host!

Tir is a central part of EPEC pathogenesis, as its deletion abolishes the actin pedestal phenotype in the cell culture infection model (Cleary 2004). Tir is expressed in the bacterial cytosol and translocated by the help of the chaperone CesT via the T3SS to the host cytosol (Abe et al. 2002). Once there, it incorporates itself into the plasma membrane in a hairpin topology with both termini inside the cell. It is known that the N-terminus is required for efficient translocation and membrane integration (Crawford and Kaper 2002) although strictly not necessary for actin pedestals (Campellone et al. 2004). Tir not only acts as a receptor for intimin, it also promotes actin polymerisation (Kalman et al. 1999; Gruenheid et al. 2001), suppresses autophagy (Xue et al. 2017), and immune response (Yan et al. 2012) to allow bacterial survival on the host cell surface. Tir suppresses the host immune response by recruiting cellular SHP-2 through immunoreceptor tyrosine-based inhibitory motifs (ITIMs). This suppression leads to a decrease in the production of inflammatory cytokines, which

helps the bacteria evade the host immune response. Tir interacts with the host cellular tyrosine phosphatase SHP-1 in an ITIM phosphorylation-dependent manner, facilitating the recruitment of SHP-1 to the adaptor TRAF6 and inhibiting the ubiquitination of TRAF6. The ITIMs of Tir suppress EPEC-stimulated expression of proinflammatory cytokines and inhibit intestinal immunity to infection with *Citrobacter rodentium*. EHEC Tir also inhibits the expression of EHEC-induced proinflammatory cytokines by inhibiting TAK1 activation, which is a key regulator of the host immune response (Zhou et al. 2019). Therefore, Tir plays a crucial role in immune evasion by these pathogenic bacteria.

Once Tir has integrated into the membrane it works as an adherence site for the pathogen, which binds to the extracellular portion of the receptor with, intimin and as a receptor tyrosine kinase sending the signal needed for actin pedestals. After the activation of the receptor with its native ligand the receptor clusters and recruits host cell actin nucleators (Campellone et al. 2004). A phosphorylation site has been described on tyrosine 474 that is essential for the canonical Tir-induced actin polymerization pathway, although there is an alternative route used in other EPEC strains (Gruenheid et al. 2001; Campellone et al. 2002; 2004; Swimm et al. 2004). This tyrosine residue (Y_{474}) on the c-terminal of Tir has been shown to be central to actin pedestal formation in the reference strain 2348/69. It is known that this residue can be located elsewhere on intimin in clinical EPEC isolates, likely due small mutation events, such as insertions and deletions of individual residues (Kenny et al. 1997). This residue interacts with the host adaptor Nck, which has a SH2-domain that binds with a 12-residue region of EPEC Tir that includes the critical phosphorylated Y_{474} (Campellone et al. 2002). SH2-domains are highly conserved eukaryotic domains that bind to phosphorylated tyrosines and consequently modifying the function or activity of the SH2-containing protein. A strictly-conserved Arg residue binds to the phospho-tyrosine in the target (in our case, Tir). The binding of the 12-residue Tir phosphopeptide to NCK then activates N-WASP and the Arp2/3 complex (Kalman et al. 1999; Gruenheid et al. 2001; Campellone et al. 2002). The Arp2/3 complex, comprising seven protein subunits, is a critical regulator of actin cytoskeletal dynamics in eukaryotic cells. By nucleating the formation of new actin filaments and promoting their branching, the complex orchestrates essential cellular processes such as cell migration, endocytosis, and the generation of cellular protrusions (pedestals).

Multiple host molecules have been shown to interact with Tir. Several tyrosine, serine and threonine residues are phosphorylated by host kinases (Kenny et al. 1997). Studies with single amino acid residue mutants seem to suggest that threonine and serine residue phosphorylation is not required for the formation of actin pedestals, but may be involved in insertion of Tir into the apical membrane (Kenny et al. 1997). Tyrosine residue phosphorylation, on the other hand, is required for actin polymerization (Kenny et al. 1997). Many kinases from Abl and Src families have been shown to colocalize with Tir in immunostaining studies. Nonspecific kinase inhibitors have been shown to inhibit actin pedestals, even though EPEC still manages to induce pedestals in knockout cell lines missing individual Src or Abl kinases (Swimm et al. 2004), suggesting that multiple kinases are at work or can complement each other.

Interestingly, a non-toxic inhibitor of EPEC pedestals was recently reported that targets this locus (Qiu et al. 2020; Bao, Qiu, and Xia 2022; Liu et al. 2023). It is known that Nck also contains three SH3 domains that are able to recruit the host actin nucleation promoting factor N-WASP and that this in turn activates the Arp2/3 complex utilized by mammalian cells to polymerize nuclear actin monomers into filaments. These protein aggregates also can be

deactivated by promoting their liquid-liquid phase separation, thus protecting cells from EPEC infection (Liu et al. 2023). However, all of these targets are intracellular, a fact that is a significant challenge for drug development, due to possible adverse effects and the need to optimize the molecular structure to gain entry to the cell.

The N-terminus of Tir is highly disordered (~52%) but forms a stable antiparallel dimer (Vieira et al. 2024). The central region of N-Tir comprises an SBB domain followed by a coiled-coil dimerization helix, thus representing a second dimerization interface in addition to the dimerization interface on the intimin binding domain of Tir in the 1F02 crystal structure. It is currently not clear whether this present preactivation with intimin or a following consequence. A hallmark of regions of intrinsic disorder is the high occurrence of so-called eukaryotic linear motifs. These are stretches of 3-10 contiguous residues mediating transient protein-protein interactions. Phosphorylation sites are often found in protein regions of intrinsic disorder, modifying charge and hydrophobicity and modulating partner interactions. Thus the roles of the various parts of the receptor for dimerization remain unknown, but there exists the possibility that the system could be modulated or interfered with via these other links in the molecular pathway, if they were fully understood.

The other terminus (C-Tir) can interact with membranes via two host-like ITIMS and its last C-terminal residues (Vieira et al. 2021). As mentioned above, it is known that Tir can inhibit innate host immune reactions via tyrosine phosphatases SHP-1/2 in a phosphorylation-dependent manner. Experiments suggest that this membrane affinity is residue-specific and modulated by both lipid composition and phosphorylation. As this happens in a quantitative and site-resolved way, such membrane interaction may be a regulation layer based on lipid composition and phosphorylation (Vieira et al. 2024). The authors speculate that transiently structured lipid-binding regions might bury tyrosine residues rendering them inaccessible, while their phosphorylation allows membrane anchoring. It remains unclear whether this would change the topology of the extracellular domain or not. The flexibility and conformational plasticity of the C-terminus could make it more readily accessible for phosphorylation.

Vieira *et al*. found in C-Tir four *bona fide* tyrosine phosphorylation sites modified by Fyn (a Src family kinase) that allow for multivalent and promiscuous binding, supporting the idea of Tir acting as a signaling hub (Vieira et al. 2024). All four of these are capable of binding the SH2-domain in the micromolar range, consistent with findings for other phosphotyrosine-containing motifs. This means that Tir may be capable of binding to multiple host proteins. One of thes sites (pY_{454}), exhibiting the lowest affinity, is situated within a conserved NPY-binding motif for the I-BAR domain of host IRSp53/IRTKS, providing Tir a connection to the actin polymerization machinery in a phosphorylation-independent manner. Y_{454} might serve as a binding site for distinct and competing host proteins contingent upon its phosphorylation status (i.e. a kind of switch). The convergence of interacting functional elements observed in C-Tir mirrors the mechanics of human intrinsically disordered domains. Tir-induced cell death has been shown to be dependent on mechanosensitive Ca_2+ channels and can be modulated by membrane mechanical stress (Zhong et al. 2020). As high density of lipid-binding proteins, such as in the case of intimin-clustered Tir, can potentially produce lateral pressure to bend the lipid membrane (Stachowiak et al. 2012).

A lot remains to be known about the exact mechanisms or Tir interaction with host cell pathways, the biophysics related to the membrane and so on. However, the extracellular

domain has a described structure and it is apparent that it interacts with intimin and that this is central to the actin cytoskeleton's interaction with EPEC. A main theme of this thesis was to target this interaction with small molecule compounds.

2.5.4 The interaction interface of Tir and intimin

According to the cocrystal structure of the extracellular domain (ECD) of intimin and the intimin binding domain (IBD) of Tir (PDB: 1F02), the interface between intimin-ECD and Tir-IBD is fairly small (around 1335 $Å^2$) for a protein-protein interaction (these are generally in the 3000 range), but still significantly larger than traditional small molecule drug binding sites (300-500 $Å^2$) and mostly flat and uncharged (Cheng et al. 2007b; Smith and Gestwicki 2012). The majority of the interface is thus involved in some way in the binding of the two domains, and the predominantly hydrophobic nature of the locus likely explains the empirically measured beneficial shift in enthalpy (Y. Luo et al. 2000). The secondary structure of intimin shows a β-sheet and a short charged α-helix at the very tip of the Tir binding domain (Batchelor et al. 2000) (Figure 8). In other words, the tertiary structure formed by the sheet and the helix form the binding location for the receptor β-hairpin. And as stated previously W899 on intimin affects the topology in a significant manner, as its deletion prevents Tir-intimin although it is buried and not at the interface.

Ross and Miller (2007) used scanning alanine mutagenesis together with binding assays to study the interactions of the individual residues on Tir with intimin. The eleven mutants they generated focused on the β-hairpin of Tir and their binding was studied using isothermal titration calorimetry. They additionally employed circular dichroism spectroscopy to identify possible folding changes caused by the mutations, as these could modify the binding characteristics for reasons other than molecular interactions between the partners. The studies found that three residues affected the binding via their chemical properties (asparagine-300, isoleucine-301, and glutamic acid-312) can be considered the binding hotspot. Other residues, such as lysine-298 and glycine-305 had profound effects on the binding likely due to structural changes caused to the type 2 β-hairpin. These two structural residue mutants caused a very large entropic penalty ($T\Delta\Delta S$ = -16.50 kcal/mol) for binding, suggesting that precise alignment and structure of the β-hairpin is very important for affinity, given that binding free energy is a function of the enthalpy and entropy. In addition, the authors speculate that since lysine-298 forms an intramolecular salt bridge, this is likely relevant to the exact geometry and position of the molecule with respect to intimin (Ross and Miller 2007). These findings underscore the fact that molecular interaction is able to achieve the level of binding that it can very likely in part because of the exact positioning of the hairpin. Asparagine-304, which in the X-ray crystal structure points away from intimin into the solvent, and valine-299, whose side chain does lie in contact with intimin but apparently does not fit into a pocket, cause almost no change when the residues are replaced with a smaller alanine.

Table 4. Hydrogen bond interactions Tir and intimin from the 2P2IDB database (Basse et al. 2016)

	Atom no.	Atom name	Res name	Res no.	Chain		Atom no.	Atom name	Res name	Res no.	Chain
1.	2860	NH1	ARG	850	I	<-->	4678	O	ILE	301	T
2.	4043	N	LYS	927	I	<-->	4648	O	VAL	299	T

3.	4051	NZ	LYS	927	I	<-->	4819	OE1	GLU	312	T
4.	4118	ND2	ASN	932	I	<-->	4668	OD1	ASN	300	T

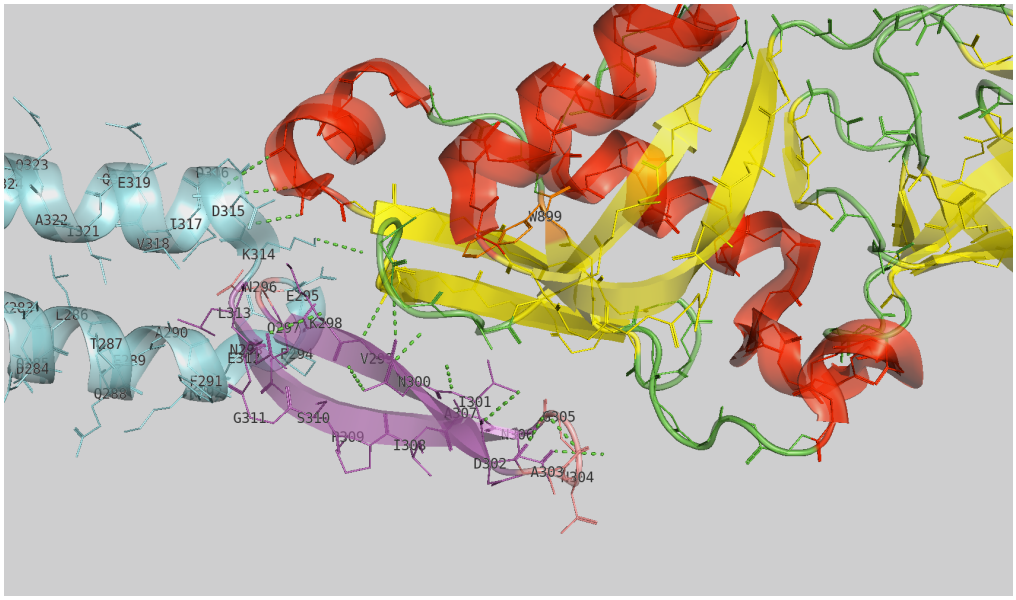


Figure 8. The interface of Tir and intimin visualized using Pymol from the PDB structure 1F02. Tir is depicted on the left side with the β-hairpin shown in a purple Ribbon. This hairpin contains several residues demonstrated to be necessary for efficient binding, such as N300 and T301. In intimin, the alpha helices are in red ribbon and the smallest one of these is seen interacting with Tir on residues D315 and K314. The buried W899 residue on intimin is indicated in orange. It has been shown that this residue is required for binding of Tir and intimin, despite it not being displayed on the interface.

Batchelor et al. studied intimin via mutagenesis (Batchelor et al. 2000). They came to the conclusion that binding was hampered by mutating residues (for example the buried W899) that do not form direct contact with Tir. Altering W899 in intact intimin prevents EPEC from forming actin pedestals by specifically disrupting the intimin–Tir interaction. This mutation lies proximal to the Tir-recognition region, which comprises a 20 × 8 Å patch of residues within the C-terminal 100 amino acids (D4). This led them to conclude that these residues might be relevant for positioning the α-helix at the tip of the intimin lectin domain (in red in Figure 8 near D315) in the correct orientation for efficient submicromolar binding of the receptor, thus creating the correct tertiary structure for the binding interaction. This, together with the fact that linear peptides of Tir hot spot do not bind intimin (Ross and Miller 2007), suggests that the tertiary shape of the interaction is relevant for efficient binding. This view is supported by other lines of evidence, for example, there are small differences between EHEC and EPEC Tir explaining why they can cross-complement each other *in vitro*, albeit with around 20 times lower efficiency (DeVinney et al. 1999; Yi et al. 2010). EHEC and EPEC Tir share most of their sequence, but the hairpin on EPEC Tir is a few amino acids longer. An avenue towards a Tir-intimin inhibitor could therefore be, for example, a stapled β-hairpin mimetic, similar to the ones already used in the design of antimicrobial peptides (Selvarajan et al. 2023). Similarly a nanobody directed towards the binding site of EHEC Tir has lower affinity towards *Citrobacter rodentium* Tir and does not inhibit EPEC Tir at all

(Ruano-Gallego et al. 2019). One can imagine that these already working structures may be possible to design into analogues that would be able to bing EPEC Tir.

2.5.5 Tir-tir dimerization

In addition to the tir-intimin interface, the intimin binding domain of Tir in the PDB 1F02 structure also displays a functional Tir-Tir interface, although much less is known about this both with respect to chemical and physical characteristics and mechanisms. The cocrystal structure PDB 1F02, shows a 1971 $Å^2$ interface, but this large size is thought to be due to crystallographic packing artifacts as it is significantly larger than expected (Y. Luo et al. 2000). The interface core is entirely hydrophobic, except for the hydrogen-bond forming Thr 282 residues on both monomers. Each Tir IBD monomer is hairpin-shaped with two long α-helices (HA, residues 274-291 and HB, residues 315-334), a three-residue 3_{10} helix initiated by Pro 294, and a □-hairpin (residues 297-311). The two α-helices mediate dimerization by forming an antiparallel four-helix bundle similar to ColE1 Rop protein, a protein responsible for plasmid copy number in *E. coli*. The Tir-tir dimer has been manipulated empirically in the laboratory, showing that it is necessary for actin pedestals to form. Indeed, Campellone et al, showed that by adding HA-tags to the extracellular domain of the receptor and expressing the construct ectopically in human cancer cells, it is possible to cause structures reminiscent of actin pedestals simply by using anti-HA antibody coated particles that then bring the receptors into proximity, that is, entirely in the absence of the natural ligand, intimin. Therefore, it can be hypothesized that the opposite may similarly be possible, that is, to inhibit the normal dimerization of the molecules by physically blocking the interface on the extracellular domain and hope that this will lead to the dimer not functioning anymore.

When Tir-IBD dimerizes, two monomers form a four helix bundle, a motif that is common in biology, especially in dimer and other multimers. Four-helix bundles typically consist of four helices packed in a coiled-coil arrangement with a sterically close-packed hydrophobic core in the center. They are typical in fibrous proteins. Because dimeric coiled-coils are themselves relatively stable, four-helix bundles can be dimers of coiled-coil pairs, as in the Rop protein. Four-helix bundles can have thermal stability of more than 100 °C. The Tir-intimin (1F02) crystal structure only displays the extracellular domains, however multiple lines of evidence suggest that Tir also dimerizes via an antiparallel oligonucleotide and oligosaccharide-binding-fold within a highly disordered N-terminal cytosolic domain (Vieira et al. 2024). It is not known, however, whether this interface is present prior to intimin binding or only upon it.

2.5.6 The auxiliary roles of the other EPEC effectors in virulence

Large mutant studies, with more than 100 mutants of EPEC murine analog, *Citrobacter rodentium*, have suggested that most of the members of the collection of EPEC effectors are dispensable in the sense that deleting them does not significantly alter the outcome *in vivo* (Ruano-Gallego et al. 2021). Many of these may be essential in a context dependent manner. In addition to the Tir and intimin being essential, also espZ and NleA (for non-LEE encoded effector A) appear to be essential (Ruano-Gallego et al. 2021). For example, EspZ is another effector encoded by A/E-lesioning pathogens that is targeted to the plasma membrane, similarly to Tir. In contrast to other effectors, it does not have any inherent cytotoxic effects. Studies show that it inhibits actin pedestals and that EPEC null mutants display higher cytotoxicity. When ectopically expressed, EspZ interferes with actin pedestal formation,

which also suggests its function is to modulate Tir induced stress to the cells (Berger et al. 2012). Perhaps it is able to do this by mitigating the previously mentioned Tir-induced mechanical membrane stress. Programmed cell death and shedding of dead cells from epithelial layers is a host protection mechanism to rid infected and damaged cells. Because the insertion of Tir into the cell and the inducing of actin pedestals likely causes the cell to detect the damage, EPEC effectors have evolved to dampen the signal to allow for longer periods of colonization.

LEE effectors EspF and Map disrupt tight junctions during EPEC infections by preventing the depletion of junctional proteins through transcriptional and post-transcriptional mechanisms. They interact with host cell proteins like ZO-1 and myosin II, promoting permeability through tight junctions independently (Singh et al. 2018). EspG and EspG2 regulate epithelial paracellular permeability in a size-selective manner during EPEC infection. EspG and EspG2 alter the function of epithelial tight junctions as channels for paracellular fluxes, contributing to EPEC-mediated diarrhea (Matsuzawa, Kuwae, and Abe 2005).

2.5.7 Inhibitors of EPEC virulence discovered from previous phenotypic screens

It is also worth mentioning that large scale phenotypic screening campaigns have suggested that A/E lesions could be prevented through other, more indirect mechanisms of action (Hotinger, Pendergrass, and May 2021). It is conceivable that many different molecular disturbances of the system could attenuate virulence in either a nonspecific way such as the obvious general toxicity, or via a method not directly related to adhesion, such as inhibiting the inducible prophages or other regulatory elements affecting effector expression or translocation thereof. The challenge with this approach is that it is difficult to determine the specific targets and mechanism of action, especially in the case of antivirulence compounds (Beckham and Roe 2014). However, various different mechanisms have been proposed to prevent T3SS mediated virulence.

The red blood cell hemolysis assay, published by Warawa et al in 1999, is one approach widely used to assess EPEC pathogenicity. EPEC causes erythrocytes to degrade, revealing hemoglobin, which may then be quantified in the supernatant using spectrophotometry. Kimura et al (2011) used this assay to screen 13,000 compounds, including some natural product libraries, and discovered compounds capable of suppressing EPEC-induced erythrocyte lysis at concentrations that would not inhibit EPEC growth. These compounds are polyketides synthesized by the actinomycete *Streptomyces goldiensis*, and were already known as they had been developed as antibiotics in the 1970's, although these projects were terminated due to lack of efficiency(Maehr et al. 1979). At concentrations significantly lower than the MIC, these compounds nevertheless protected mice from *Citrobacter rodentium* infections. Mice were initially infected with *C. rodentium*, then treated either with 10% dimethyl sulfoxide as a control, a single dose of tetracycline (200 mg/kg), or 25 mg/kg aurodox every 24 hours for four days. All of the mice that were treated with aurodox survived while none of those that were treated with tetracycline survived past day 13. The compound with the highest efficacy, aurodox, was later examined further using RNA-seq to indicate that it likely acts by inhibiting a master regulator called Ler (McHugh et al. 2021). Much of the LEE was found to be downregulated, including transcription of the master regulators Ler and GrlA. Their findings also show that removing T3SS system components like structural proteins does not result in transcriptional feedback on LEE1. In conjunction with the

transcriptomics data, they theorize that aurodox binds some target that modulates Ler transcription, but it remains to be shown exactly what that is.

Another screening campaign similarly using large collections of compounds from various sources, including bacterial metabolites discovered compounds that can prevent the translocation of EPEC effectors (Mühlen et al. 2021). The authors used a betalactamase reporter fused to Tir as a proxy of translocation efficiency. Some of the compounds were able to inhibit this measurement without affecting growth orcausing the release of shiga toxins. The authors ponder that the effect is likely not caused by the down regulation of expression of the LEE effectors as their concentrations in the supernatant do not decrease when inspected by Western blotting. They then speculate that there must be some other mechanism of action by which the compounds are able to prevent the translocation of Tir and thus decrease the fluorescence readout from the reporter. However, the mechanism of action or possible target of these compounds remain unknown, and no further studies have been completed on them since their original discovery

The large glycolipid caminoside A was found in extracts of the marine sponge *Caminus sphaeroconia* and was the first T3SS inhibitor ever described. It decreased the secretion of the EPEC translocon component EspB into the supernatant without affecting bacterial growth or the secretion of other proteins (Linington et al. 2002). Here again, it is not known by what mechanism of action these compounds work and these compounds are difficult to acquire. A total synthesis was published in 2005, but consists of 57 steps, and thus has not entered into the catalogues of chemical vendors (Sun, Han, and Yu 2005).

The guadinomines were discovered by Ōmura and colleagues using EPEC-mediated hemolysis to screen natural product extracts(Iwatsuki et al. 2008). *Streptomyces sp.* K01-0509 was found to produce potent inhibitors of RBC hemolysis Cultures were scaled up, and guadinomine A, B, C1, C2, and D were isolated, purified and analyzed (Iwatsuki et al. 2008)). Guadinomines A and B are the most potent natural product T3SS inhibitors (IC_{50} = 0.007~0.01 μg mL^{-1}). The mechanism of action of the guadinomines is not well understood and yields of guadinomines from culture are low, making further research difficult. The lengthy total syntheses of guadinomine B and C2 have been published, with 33 steps in the longest linear synthetic sequence (Hirose et al. 2008). In 2012, a study on the biosynthetic pathway of guadinomine A was published (Holmes et al. 2012).

These examples suggest that natural products, especially of other microbial entities, can possibly induce or suppress metabolic states in which the pathogen is virulent. However, getting ahold of these compounds may be difficult and screening for them may be easier starting from recognizing the biosynthetic gene clusters and producing them via heterologous expression. Unfortunately, oftentimes natural products that are described to have bioactivity in early in vitro screening assays are never researched further.

3 Aims of study

EPEC initiates abnormal cytoskeletal changes in infected cells by binding to the translocated cell membrane penetrating receptor Tir. Inhibiting this process may prevent EPEC virulence and therefore the aim of this study was to search for compounds capable of inhibiting Tir, or its ligand, intimin. The specific aims of this study were:

1) To set up, evaluate, and validate a high-throughput compatible screening assay of the fluorescent actin staining (FAS) assay (I)
2) To use virtual screening methods (docking studies) to search for compounds capable of inhibiting the interaction of Tir and intimin, and then to test these compounds using the FAS assay (II)
3) Testing previously unstudied marine bacterial extract for their capacity to reduce EPEC-based virulence (III)

4 Materials and Methods

The reagents, materials, and methods used in the thesis are detailed in the following section. The description provides a basic overview of the methodology used; more specific techniques, materials, and methods can be found in the original articles (I-III).

4.1 Bacterial strains and culture conditions

A typical EPEC type 1 strain, *Escherichia coli* 2348/69, was obtained from Reference Microbiology Services in the United Kingdom. Single colonies from a weekly LB agar culture were grown in liquid overnight cultures in LB broth at 37 °C and 200 rpm.

To facilitate image acquisition, this strain underwent transformation with a plasmid expressing mCherry constitutively, which was originally deposited to Addgene by Howard Schuman (pON.mCherry, Addgene #84821)(Gebhardt, Jacobson, and Shuman 2017). The plasmid was extracted using a miniprep kit (GeneJET, Thermo Fisher Scientific K0502). Following extraction, chemical transformation was used (rubidium chloride method), and transformants were selected using 30 µg mL^{-1} chloramphenicol.

For Tir translocation studies a Tir betalactamase reporter fusion strain was gifted to us from the lab of Ilan Roshenshine (Hebrew University of Jerusalem). This was cultured in LB broth or agar supplemented with 30 µg mL^{-1} tetracycline.

For assessing the metabolites of the marine actinobacteria, the four strains (Table 3) were cultured in modified marine ISP2 medium. These strains were collected earlier on a scientific cruise around the Svalbard area (Schneider et al. 2022).

Table 5. Actinobacteria isolates investigated. More detail on the field sites and methodology used to isolate and culture the strains can be found in (Schneider e 2022)

Strain ID:	T091	T289	T060	T160-2
Animal/Origin	*Caulophacus arcticus*	*Chlamys islandica*	*Dendrobeania sp.*	*Tricellaria ternata*
Genus	*Kocuria sp.*	*Rhodococcus sp.*	*Rhodococcus sp.*	*Rhodococcus sp.*
Location/depth	77.37491402 °N 8.268010617 °E 1400 m	74.78804448 °N 18.57096443 °E 285 m	75.16057145 °N 13.71765962 °E 1500 m	75.16057145 °N 13.71765962 °E 1500 m

4.2 Computational modeling and virtual screening

A 2.9 Å resolution crystal structure of the C-terminal 282-residue fragment of intimin in complex with the intimin-binding domain of translocated intimin receptor (PDB code 1F02) was used for all the models. Three different models were designed as follows.

4.2.1 Compound libraries

Drug-like small molecule libraries from Asinex, ChemBridge, Enamine, Life chemicals, Key Organics and Vitas-M were downloaded in SDF format. These libraries were merged and duplicates removed, which resulted in a library containing 741,778 compounds. For these compounds a library of conformers was generated using the OMEGA software (Release 4.1.1.1, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com) using default settings, which resulted in a maximum of 200 conformers per ligand.

A collection of compounds available at the Institute for Molecular Medicine Finland (FIMM), University of Helsinki, (FIMM) in a database of 132566 compounds was prepared using LigPrep by including options such as adding hydrogens, generating ionization states at pH 7.2 ± 2.0, generation of tautomers, and multiple conformers.

4.2.2 Structure-based virtual screening

Model 1: Inhibiting Tir-intimin interaction on the binding site of intimin on Tir

The binding site was prepared from the PDB 1F02 data using MAKE RECEPTOR (Release 4.1.0.1, OpenEye Scientific Software, Inc., Santa Fe, NM, USA). A grid box around the Asn300 residue of Tir was generated and adjusted to the following dimensions: 17.33 Å x 26.33 Å x 14.67 Å and a volume of 6694 $Å^3$. For "Cavity detection" the slow and effective "Molecular" method was used. Inner and outer contours of the grid box were also calculated automatically using the "Balanced" settings for "Site Shape Potential" calculation but the inner contours were disabled. Asn300 was defined as a hydrogen-bond donor and acceptor constraint for the docking calculations. A small molecule library, prepared by OMEGA, was then docked to the prepared binding site on intimin (PDB entry: 1F02) using FRED (Release 4.1.0.1. OpenEye Scientific Software, Inc., Santa Fe, NM, USA). Docking resolution was set to high and other settings were set as default. A hit list of top 1000 ranked molecules was retrieved and the best ranked FRED-calculated pose for each compound was inspected visually and used for analysis and representation.

Model 2: Intimin extracellular domain

Structure-based virtual screening

The protein preparation workflow of Maestro Suite was used to prepare the intimin protein domain, by separating it from the cocrystal structure (1F02), correcting errors in the structure, such as adding missing hydrogens and minimization. For the binding site preparation, the above mentioned crystal structure was used and a grid box was created . Glide grid

generation was performed around the alpha helix (residues 904-909) of intimin with dimension 20 Å x 20 Å x 20 Å.

Docking studies were conducted to pinpoint potential interacting ligands utilizing the Glide virtual screening workflow, which is integrated into Schrödinger. This workflow encompasses multiple stages with escalating docking accuracies, namely high-throughput virtual screening (HTVS), standard prediction (SP), and extra precision (XP) mode. Initially, all molecules underwent processing in HTVS mode, and the top 10% of HTVS output underwent further processing with SP docking mode. Subsequently, the top 10% of molecules from SP docking were subjected to XP docking.

Model 3: Inhibiting Tir dimerization on the ECD

The protein preparation workflow within the Maestro Suite was employed to ready the intimin protein domain, accomplished by isolating it from the cocrystal structure (1F02), rectifying structural inaccuracies such as adding absent hydrogens and conducting minimization. Subsequently, Schrödinger SiteMap was utilized with default settings utilizing the PDB entry 1F02, following the extraction of intimin-ECD and its subsequent removal (so that only the receptor remains). For shallow sites, specific settings were configured accordingly. However, the Glide grid was established based on the site identified using default settings, positioned adjacent to the Trp285 residues involved in hydrogen bond formation within the dimer interface.

Using the Glide virtual screening workflow built in Maestro, docking tests were performed to identify potential interacting ligands. All molecules were originally processed in HTVS mode, with the top 10% of HTVS output being processed in SP docking mode. The top 10% of SP docking molecules were then processed through XP docking.

4.3 Bioactivity screening

4.3.1 High-throughput fluorescent actin staining assay

Primary screening and concentration response experiments were performed using a 384-well plate modification of the widely used FAS assay published earlier. The EPEC strain 2348/69 was modified to constitutively express mCherry, a fluorescent protein using a construct acquired from Addgene (See section 4.1). The screening plates were prepared 24 hours before the assay by seeding 2×10^5 Caco-2 (ATCC CCL-23) cells mL^{-1} into black 384-well plates with transparent bottom (Phenoplate, Perkin Eelmer, Germany)

To screen the compounds, a MEM preincubated EPEC-mCherry suspension was added to the above source plates using a dispenser (Mantis, Formulatrix) in such a way that in the end the source plate contained the correct concentration of the compounds and a bacterial suspension with a multiplicity of infection of 1:15. A volume of 60 µL of this mixture was moved to the screening plates plated with Caco-2 cells and these plates centrifuged at 1000 g for four minutes, in order to bring the bacteria into contact with the cells, and incubated for two hours at 37 °C, 5% CO_2 and 95% humidity (Biospa, Biotek). After this, a PFA-containing staining solution was applied to the plate using a dispenser (Mantis, Formulatrix) and incubated at RT for 20 minutes. The plate was then washed with HBSS with a pipetting robot (Biomek i7,

Beckman Coulter) and imaged using a protocol in an imaging station (Cytation 5, Biotek). The images were analyzed using custom Python scripts on a high-performance computing cluster.

4.3.2 Tir translocation assay

For translocation assays, 2×10^5 Caco-2 cells mL^{-1} (ATCC CCL-23) were seeded into black 384-well plates with transparent bottom (Phenoplate, PerkinElmer, Germany). Bacterial overnight cultures were diluted 1:50 into MEM with GlutaMAX (Gibco, Germany) and incubated for 2 h at 37 °C 5% CO2 in a filter capped 50 mL Falcon tube. 100 µL of the bacterial suspension was subsequently added to a source plate (96-well, NUNC) to which samples were serially diluted. These plates were incubated for an additional 1 h. Caco-2 cells were washed once with HBSS. The bacteria-sample suspension (60 µL per well) was then added to the cells, the plates were centrifuged at 1,000 × g for 4 min (Eppendorf Centrifuge 5810R with a A-2-DWP-AT plate rotor) and incubated for 1.5 h (37 °C, 5% CO2). Media was then removed, and infected cells were washed twice with 100 µL HBSS. MEM with 100 μgmL^{-1} gentamicin was added to the cells and mixed with LifeBLAzer CCF4-AM staining solution (Invitrogen). The plates were then incubated for 1 h at room temperature. Subsequently, the fluorescence was determined in a Cytation 5 (Biotek, Germany) using an excitation wavelength of 405 nm (10 nm bandwidth). Emission was detected with 460 and 530 nm. Effector translocation was determined by calculating the ratio of blue to green fluorescence (Em520 nm/Em460 nm) following the manufacturer's instructions.

4.3.3 Growth inhibition studies

EPEC 2348/69 was grown overnight in LB liquid culture. Compounds were plated onto a 384-well plate in LB or Miller Hinton broth and shaken (500 rpm, 30 min) using a Biomek i7 protocol. The bacterial suspension was then diluted to the appropriate range (2 million CFU mL^{-1}) by measuring the optical density using a densitometer. This suspension was dispensed using a dispenser (Mantis, Formulatrix) into the compound plate to reach a total volume of 75 µL. After this, the plates were incubated using Biospa (Biotek) for 24 hours and their OD_{600} measured once an hour using Cytation 5. This procedure was repeated three times using a different colony and newly prepared media and other reagents, and the well location of a specific compound series was varied among the repeats randomly to diminish any possible plate area effects.

4.3.4 Assessment of cellular viability

Cellular viability was assessed using the CellTiter-Glo (Promega) kit according to the manufacturer's instructions. Assay conditions were identical to the screening procedure described above except that 0.5% Triton-X was used as the positive control and PBS with 1% DMSO as the negative.Three independent experiments were performed on distinct days using newly acquired media and reagents.

4.3.5 Red blood cell hemolysis

EPEC overnight cultures were diluted 1:25 in DMEM high glucose without phenol red (Gibco) and grown for 3 h at 37°C, 5% CO2 in the presence or absence of decreasing concentrations of the inhibitors. Red blood cells (RBC) were purified from Defibrinated

Oxoid Sheep Blood (Thermo Scientific) by three rounds of centrifugation and washing with PBS, then resuspended to 5% (vol/vol) in DMEM high glucose without phenol red. Bacterial cultures were equalized to 1 × 108 in 100 µl and added to 100 µl sheep RBCs (5% vol/vol) in a 96-well plate. Uninfected RBCs in DMEM were used as a negative control. Total lysis was achieved by adding 0.5% Triton-X to the culture medium. To synchronize infection and mediate bacterial-cell contact, tubes were centrifuged 1 min at 3220 × g before incubation at 37°C, 5% CO_2. After 2 h, cells were gently resuspended, followed by centrifugation at 3220 × g for 1 min. 50 µl of each supernatant was transferred to a 96-well plate, and the amount of hemoglobin released was assessed at 543 nm in Cytation 5 (Biotek) plate reader. Hemolysis was calculated as the percentage of hemoglobin released by the DMSO-treated wild-type-infected red blood cells.

4.4 Bioprospecting methods

4.4.1 Extraction and purification of marine bacterial extracts

Purified H_2O was produced by the in house MilliQ system, Methanol (HiperSolv, VWR, Radnor, PA, US) and Acetone () where used if not otherwise indicated.

Modified marine ISP2 medium was produced using 4.0g glucose (Sigma Aldrich, St. Louis, MO, US) 4.0g yeast extract (Sigma), 10.0g malt extract (Sigma). 300 mL filtered seawater, 700 mL dH_2O and 0.2% (v/v). Filtered seawater was produced by the seawater supply of the Norwegian college of fishery science in Tromsø, Norway by filtration through a 0.22µm Millidisk® 40 filter-cartridge (Millipore, Billerica, MA,US). The trace element solution was prepared by dissolving 10% $MgSO_4$ 7 × H_2O, 0.01% $FeSO_4$ 7 × H_2O, 0.01% $ZnSO_4$ 7 × H_2O, 0.01% $CuSO_4$ 5 × H_2O, 0.01% $CoCl_2$ 6 × H_2O in pH_2O. The media was autoclaved at 121°C for 30 min using an inhouse autoclavation system.

Crude extracts were fractionated using flash liquid chromatography. The extracts were loaded onto resin (Diaion® HP-20ss, Supelco) by dissolving them in 90% methanol aq. (v/v) and adding resin in a ratio of 1:1.5 (resin/dry extract, w/w). Subsequently, the solution was dried under reduced pressure at 40 °C. Flash columns (Biotage® SNAP Ultra, Biotage, Uppsala, Sweden) were prepared by activating the resin by incubation in methanol for 20 min, washing with ddH_2O, and loading it into the column ensuring the resin being always covered with water. 6.0 g HP-20ss resin was loaded on one column. The fractionation was performed using a Biotage SP4™ system and a water: methanol gradient from 5–100% methanol over 36 min (6 min 5% B, 6 min 25% B, 6 min 50% B, 6 min 75% B, 12 min 100% B) followed by a methanol: acetone step-gradient (4 min methanol, 12 min acetone). The flow rate was set to 12 mL/min. 27 eluent fractions to 24 mL each were collected in glass tubes and pooled to six flash fractions in total (1–3 were pooled to fraction 1; 4–6 to fraction 2; 7–9 to fraction 3; 10–12 to fraction 4; 13–15 to fraction 5; 16–27 to fraction 6). An appropriate amount of extract-resin mixture was loaded onto the column after equilibration to 5% methanol aq. (v/v). The flash fractions were dried under reduced pressure at 40 °C.

4.4.2 Fractionation and analysis by liquid chromatography and mass spectrometry

FLASH fractionation of the extracts was executed by FLASH-liquid chromatography using HP-20SS resin as mobile phase and a Water:Methanol:Acetone step gradient as described in (Schneider et al. 2022) under “4. Material and Methods” section “4.4. Fractionation”.

For HPLC-HR-MS2 analysis a Acquity I-class UPLC (Waters,Milford, MA, USA) was used coupled to a PDA detector and a Vion IMS QToF (Waters). The HPLC was equipped with a Acquity C-18 UPLC column (1.7 µm, 2.1 mm × 100 mm) (Waters). The mobile phases consisted of acetonitrile (HiPerSolv, VWR) for mobile phase A and pH2OO as mobile phase B, both containing 0.1% formic acid (v/v) (33015, Sigma). The gradient was run from 10% to 100% B over 13 min at a flow rate of 0.45 mL/min. Samples were run in ESI+ and ESI- ionization mode. The data was processed and analyzed using UNIFI 1.9.4 (Waters). Exact masses were calculated using ChemCalc (Patiny and Borel, 2013). For dereplication an extract of modified marine ISP2-medium and flash fractions of the extract, where prepared and analyzed using the same HPLC-MS2 method in order to exclude media components from consideration. i) PubChem and ii) Chemspider where used to identify potential compounds during dereplication by elemental composition search (i+ii) and MS-fragment search (ii, implemented in UNIFI)

4.4.3 Isolation using RP-HPLC-MS via mass triggered fractionation

For the Isolation of compounds from flash fractions preparative reversed phase HPLC was used. Fractionation was triggered by the recorded mass signal throughout the chromatographic separation. The HPLC system consisted of a Waters 600 HPLC-pump with a degasser and flow-splitter, a Waters 515 HPLC-pump as a “make-up” pump, a Waters 3100 Mass detector , a Waters 2996 photo array detector and a Waters 2767 sample manager (all Waters). The system was controlled using MassLynx V4.1 (Waters) software A Sunfire RP-18 preparative column (10 µm, 10 mm × 250 mm) and XSelect CSH preparative Fluoro-Phenyl column (5 µm, 10 mm × 250mm) (both Waters) were used as solid phases for the first and second round of purification, respectively. The mobile phases for the gradients were A [pH_2O with 0.1% (v/v) formic acid] and B [acetonitrile with 0.1% (v/v) formic acid], flow rate was set to 6 mL/min. Acetonitrile (Prepsolv®, Merck KGaA, Darmsatdt, Germany) and formic acid (33015, Sigma) were purchased in appropriate quality, ddH_2O was produced with the in-house Milli-Q® system. For the MS-detection of the eluting compounds one percent of the flow was split and blended with 80% MeOH in pH_2O (v/v) acidified with 0.2% formic acid (Sigma) and directed to the ESI-quadrupole-MS. The fractions were collected by mass triggered fraction collection and the respective fractions were reduced to dryness under reduced pressure and by vacuum centrifugation, both at 40°C.

5 Results and discussion

This chapter summarizes the results of the publications I-III. Publication I describes the study of a classical microbiological assay and describes its performance using an automated microscopy station with a data-analysis pipeline that segments the images and measures actin pedestals. Publication II was focused on exploring possible chemical structures capable of inhibiting the translocated intimin receptor of EPEC using computational methods (docking) and the above-mentioned screening assay to probe the compounds selceted from the modeling. Study III tested metabolites of four Arctic marine actinobacteria for EPEC

virulence inhibiting properties. These cultures were solid phase extracted, fractionated and these fractions screened for their capacity to inhibit EPEC virulence using multiple assays.

5.1 The FAS assay can be performed in multiwell plates with imaging stations

The *in vitro* study of EPEC virulence was made possible by the invention of the fluorescent actin staining (FAS) assay by Stuart Knutton et al. in 1989. In this work Caco-2 cells were infected with multiple EPEC strains, including 2348/69, on microscopy slides and phalloidin-fluorescein was used to visualize the resulting actin pedestals for fluorescence imaging. Importantly, it also established and optimized the parameters and kinetics for the assay, showing that pedestals are at their strongest at the 3 hour mark on Caco-2 cells, and that the infection begins to dissipate after 6 hours, with bacteria leaving the microcolony. Additionally, this work truly validated the methodology by characterizing the effects of several other EPEC and non-EPEC A/E strains in this assay and showing that 80% of EPEC strains (including the 2348/69 strain) adhere to Caco-2 monolayers.

Today imaging stations for multiwell plates are common and allow for the studying of hundreds of compounds on a single assay plate. In publication I we were able to show that such an imaging station (Cytation 5, Biotek) can be successfully used to perform the FAS assay in a 96-well plate, so that it could be possible to test large collections of candidate compounds in a comparable way. We followed a method typically used in imaging EPEC infections, that is, by utilizing a plasmid encoding a constitutively expressed fluorescent protein. After capturing images, we segmented the images based on these fluorescent bacterial colonies and analyzed the actin signal from the equivalent cellular loci (I: Fig. 2). Today, a more popular choice would have been the use of semantic segmentation and analysis, with deep learning (i.e. Mask R CNN directly on the actin channel) without altering the genetics of the model pathogen, but T3SS inhibiting compounds and mutations also decrease adhesion of the bacteria altogether in addition to inhibiting actin pedestals, and therefore a fluorescence signal from the bacteria can be useful to quantitate the size and count of microcolonies for deconvolution of different biological effects (i.e. prevention of initial attachment, colony forming, or intimate attachment and so on). Obviously, a severe weakness of this approach is that the initial segmentation is dependent on an unstable property, that is the microcolony, as we cannot know how the size of the microcolony affects the final readout. We also added the LEE transcription inhibiting compound aurodox (at 5 $\mu g\ mL^{-1}$) as a positive control (I: Fig. 3). Using aurodox in such a manner is suboptimal, as its mechanism of action differs radically from desired direct orthostatic inhibitors of Tir-intmin, as it indirectly works on the regulators of the expression of LEE. In practice, to achieve an effect, aurodox needs to be added to the bacterial culture several hours earlier than the tested compounds, which means that the bacterial culture in the control condition is not identical to the culture used in the testing of the compounds on the screening plate. The approximate count of bacteria in these distinct cultures was attempted to control for by using a densidometer, however.

Our research findings indicate minimal variation between the 96-well plates (I: Fig. 4). Neither the Caco-2 cells nor the 2348/69 strain showed any signs of damage from DMSO concentrations up to 5%. Furthermore, we discovered that classical computer vision algorithms from the 1980s, such as the Otsu thresholding and Canny transform implementations from the Python library, scikit-image, can effectively segment and annotate

images of fluorescent EPEC microcolonies and actin pedestals automatically. This enables the automatic counting of objects and quantification of their size and other properties.

One of the large issues in pharmaceutical research is the lack of disease relevant *in vitro* models. Because of this it is many times difficult to "translate" findings from *in vitro* research to real clinical applications. In the case of EPEC infections, it is not entirely clear how well cell monolayer infection models and actin pedestals are associated with disease (Gad Frankel and Phillips 2008). This is part due to the fact that the exact mechanisms of EPEC pathogenesis are not known (Lapointe, O'Connor, and Buret 2009). But in addition, interesting differences exist between the monolayer model, and *ex vivo* transplants or biopsies (Gad Frankel and Phillips 2008). This challenge can be mitigated in part by the simultaneous use of multiple orthogonal screening designs, such as the ELISA, RBC lysis and effector-reporter fusion approaches to the extent that resources allow. In addition to this, however, higher-throughput models recapitulating any of the normal histological and functional properties of the intestinal barrier will be of help in the future.

Despite there being no publications describing compounds capable of inhibiting EPEC pedestals, there are many compounds described to be able to prevent A/E pathogenesis in some way (T. J. Ochoa et al. 2003; Theresa J. Ochoa, Noguera-Obenza, and Cleary 2004; McHugh et al. 2018) and thus it is not clear to what extent the monitoring of actin pedestals in cell culture is or is not a disease-relevant model. While aurodox shows a minor signal in pedestal inhibition in the FAS assay, it nevertheless caused better protection against EPEC lethality than standard antibiotics *in vivo* (Kimura et al. 2011). Tir-induced actin polymerization also occurs significantly later than the onset of symptoms upon ingestion of EPEC. This suggests that the primary causative agent of diarrhea is unlikely to be actin pedestals, but perhaps ion disbalances and inflammation caused by the bacteria (Kaper, Nataro, and Mobley 2004; Kaur and Dudeja 2023). Despite this, compounds successfully inhibiting actin pedestals could possibly decrease the severity of the symptoms, or alleviate chronic infections, by eradicating a disease promoting niche in a part of EPEC infections which in turn could help to save lives. In addition, studies have indicated that Tir mutants and atypical EPEC wildtype strains, while missing essential factors for virulence, nevertheless express other ones, such as the cytotoxin espC.

In the original publication we decided to not report on the limits of the assay using simulated data to test the limits of the method. In my opinion, this is a very central and important property to characterize. In a test of this 20 real life masks were selected from images of Caco-2 infected with mCherry-EPEC at a MOI of 15. These masks were then applied to images of uninfected, stained Caco-2 monolayers and processed as normal. These results indicate a 0.2 readout on average, which should be considered the lower limit of the assay. This is mostly likely caused by the fact that the labeled actin is at times spontaneously present in uninfected cells under colonies in a manner similar enough to be segmented as a pedestal by the algorithm. The upper limit is on average 0.85 colonies with pedestals, which is coherent with reports from the literature.

5.2 Classical docking studies do not reveal druggable binding site nor small molecule inhibitors in EPEC Tir-intimin

Luo et al. presented a crystal structure in 2000 that showed the extracellular domain of the translocated intimin receptor bound to intimin's Tir binding domain. This structure also indicated that the receptor's extracellular domain forms a dimer, which was later validated experimentally (Campellone et al. 2002; Vieira et al. 2021). Thus, we hypothesized that the normal function of Tir may be inhibited by either interfering with the Tir-intimin interface or alternatively by blocking Tir clustering at the extracellular dimer interface.

In study II, SiteMap was used to analyze the structures and unfortunately, no clear druggable binding pockets or clefts are present in the interface, nor in the entire structure for that matter. Indeed only one small site was present on Tir at all, and this had a low Dscore of 0.46, although it appeared to be at one of the dimerization interfaces. In the original publication, SiteMap authors suggest that sites with a score of less than 0.8 are undruggable, and high quality sites will typically achieve scores above 1.0 (Halgren 2007). The main component contributing to this low score was the extremely small size of the pocket (roughly 11 $Å^3$). Pocket size is known to be the best predictor of a site's druggability. Average drug binding site sizes are in the range 100-300 $Å^3$ (Halgren 2007). In addition, the discovered site does not really display enclosure, and thus the putative compounds cannot be well protected from the solvent phase. Decreasing the requirement for enclosure was used to search for shallow pockets in the Maestro (Schrödinger) implementation of SiteMap, of which one was located at the same locus as the initially discovered site. This shallow site had a better Dscore of 0.99, but such shallow sites are generally believed to be better for macromolecular binding.

In addition, docking was used to test these compounds on a grid placed on a locus of intimin (residues 900-930 between the β-sheet under W899 and the α-helix at the tip) that has been shown to interact with Tir (discussed in chapter 2.3.2). And lastly, virtual screening was performed on a site constructed by collaborators around the Tir-intimin binding site residues on Tir (amino acid residues 300 and 310 on Tir) using OpenEYE software.

The virtual screening procedure for the first two models was conducted using an in-house library from FIMM containing around 132566 compounds. The best predicted compound poses had estimated binding energies less than -8 kcal mol^{-1}. This predicts low probability of binding and the manual of Glide suggests to use -10 kcal mol^{-1} as a minimum for interactions to be more than just noise. However, any minimum cutoff cannot be empirically validated or challenged, and many publications utilize even worse scores. It is important to remember that docking scores (unlike free energy perturbation calculations) cannot be used to predict real life binding affinities, and that top ranking hits typically do not provide the best leads, although the overall prediction of docking may work fairly well in some specific cases, e.g. they can capture a native pose for an actual binder. The third model was used to virtually screen for compounds in multiple publicly available commercial collections and inhouse compounds at the University of Ljubljana.

We then acquired and experimentally tested 72 of these compounds using a 384-well plate adaptation of the FAS-HCS-assay described in study I. The compounds for the first model were tested at 50 µM and 25 µM with four technical replicates, the compounds for the second at 50µM (three technical replicates) and the compounds for the last model at varying concentrations between 1-200 µM with two technical replicates but three biological replicates. Six compounds from the first screen, based on the first model, reduced the readout. One compound reduced the readout from the second model, but this turned out to be an artifact due to the compound being fluorescent. None of the compounds from the third model showed activity at concentrations up to 200 µM.

Of the six compounds giving positive readouts in the screen, a few were cytotoxic or not available from commercial suppliers, thus three of the compounds were selected for further study. This involved FAS-assay at additional concentrations, growth inhibition assays, and the use of a cell viability kit. All of these compounds (at 50 and 25 μM), despite inhibiting pedestals, demonstrate possible toxicity towards the bacteria in the form of inhibition of growth and changes in colony formation and other properties through unknown pathways and mechanisms of action. Whether the pedestal inhibiting effect of the screened compounds was caused by decreased viability, binding and inhibiting Tir or some third option (or some combination of these), remains unknown. Some of the compounds even significantly increased bacterial counts in the treatment conditions versus control, despite being growth inhibitory. However, for those compounds that did inhibit growth, this occurred at such high concentrations (i.e. growth inhibition of 90% at 50 μM) that they were not considered interesting antimicrobial leads.

Much more research can be done in this area, particularly by employing strategies that have previously been successful in the discovery of protein-protein inhibitors. One avenue for deeper exploration of Tir-intimin would involve employing an empirical approach that is more closely aligned with the modeling endeavor's objectives. Modeling, fundamentally, aims to forecast the binding of small molecules to a target protein, such as Tir or intimin in this case. However, conventional low-resolution microscopy-based methods, such as the FAS-assay applied in this study, fall short in providing insights into the specifics of how, and to what extent, molecules bind to target proteins, and cannot therefore be used to verify or challenge the computational model. Although virtual screening can be used to attempt to enrich the collection of screened compounds with relevant structures, this generally works when some true binding mode or pose is known. Conversely, it is logically true that, in the worst case, an erroneous modeling methodology may even actively decrease the frequency of truly active compounds from the collection to be screened.

Indeed, even the nature of any correlation between such small molecule binding to Tir or intimin and the resulting phenotypic effects is hard to predict and unknown. For instance, any particular compound's interference with Tir-intimin interactions may not exert a potent or long lasting enough influence to bring about significant macro-phenotypic alterations (eradicate actin pedestals) despite the compound binding the model locus. While it is conceivable that molecules exhibiting weak or transient binding to the target could be subject to optimization to enhance their Tir-binding capacity, the preliminary identification of such compounds is a pivotal first step not possible to achieve with crude phenotypic methods alone. However, various biophysical methods and binding assay types exist that could provide such data or alternatively, perhaps qualitative differences in the actin dynamics could even be uncovered with imaging methods of higher resolution, for example.

This is particularly apparent in the context of fragment-based methods employed for discovering inhibitors of protein-protein interactions. Typically, these fragments exhibit exceedingly weak binding affinities. Nevertheless, this information has been effectively harnessed to engineer larger molecules from multiple fragments with improved binding characteristics. In practice, the domains of Intimin and Tir, as generated by Lou et al. (2000) and detailed in the PDB entry (1F02), could be expressed and purified using the methods outlined in their work. Subsequently, these proteins could be utilized in conventional binding assays or using biophysical methods to advance our understanding of their interactions. Alternatively, whole cell biophysical methods such as CETSA MS or similar proteomic

methodologies could be used to examine e.g. thermal shift upon binding in infected or transfected living cells. Similarly, in essence, due to this lack of the empirical method to falsify the computational model, it is likely that the models were simply wrong although we do not have data to prove this.

Another area for improvement revolves around the coverage of chemical structure space. It has been well-established that commercially available chemical libraries primarily consist of simplistic two-dimensional structures that lack chiral centers (Feher and Schmidt 2003). Given that the majority of compounds effective in inhibiting protein-protein interactions (PPI) do not conform to these characteristics (Shangary and Wang 2009; Voter and Keck 2018), specialized libraries enriched in compounds suitable for PPI have been devised (Milhas et al. 2016). Many chemical supply companies offer dedicated PPI collections tailored for this purpose. Furthermore, both LEE downregulators and Tir translocation inhibitors were discovered in large (more than 10,000 compounds) natural product collections that contain structurally more diverse compounds. In contrast, the compounds screened in this work for the most part do not contain either of these structural properties, despite there being so-called "diversity" (i.e. diversity oriented synthesis campaign) collections in the FIMM library. Moreover, due to limitations in resources, the compounds scrutinized empirically in this study constitute just a minuscule fraction, e.g. approximately 0.005%, of the FIMM chemical library, and thus is unlikely to be a representative sample of the structural (scaffold) diversity offered by the collection, which in itself is well representative of existing available compounds on the market. Consequently, it is conceivable that this limited subset might be missing true Tir-intimin inhibiting compounds possibly present in the full FIMM collection, and that it is nor a representative sample of existent chemical diversity. Subsequent research efforts could consider expanding the exploration of chemical diversity, potentially by conducting random screenings from more extensive and heterogeneous chemical libraries, or using fragments which are known to be able to represent a larger chemical structure space with smaller libraries. It is noteworthy to mention that, to date, there are no documented instances of successful design of protein-protein interaction inhibitors through the amalgamation of target-based virtual screening (docking) and low resolution phenotypic assays to my knowledge.

5.3 Metabolites from marine actinobacteria for preventing EPEC virulence

Many of the most successful protein-protein interaction inhibitors are from natural product sources (Marston 2011). In addition, many compounds inhibiting EPEC virulence have been discovered in bacterial metabolite libraries (Kimura et al. 2011; Mühlen et al. 2021). We therefore decided to screen metabolites of previously uncultured marine actinobacteria for EPEC virulence inhibition. We used a collection of four bacteria, collected by colleagues at the Universitet i Tromsø (Schneider et al. 2022), using three different screening approaches 1) the actin staining assay, 2) a Tir translocation reporter assay and 3) sheep red blood cell hemolysis. The translocation assay is an approach, developed in 2004 by Charpentier and Oswald, in which one C-terminally tags the effector with the TEM-1 β-lactamase and then infects CCF2-loaded cells (Charpentier and Oswald, 2004). The compound (CCF2) is a FRET fluorophore with the acceptor and donor connected via a betalactam, and thus a signal shift occurs if the molecule is cleaved by betalactamase from the Tir-fusion protein. Previous to this, it was not known whether or not these assays can be utilized with natural product fractions, as fractions can easily contain interference compounds.

Two of the metabolite crude fractions caused changes in the assay readouts, but one of these – from the *Rhodococcus* strain, T-160-2 – also inhibited growth in broth microdilution assays, leading us to believe that it contains a compound with antibiotic-like toxicity (III figure 3) to cells in general. However, the fraction from the *Kocuria* sp. strain, T-095, minorly changed actin staining readouts without any effect on growth nor formation of microcolonies in the infection model. We do not know at what concentration these compounds were present in the fractions.

These fractions were further studied by subfractioning the crude fractions displaying activity into smaller subfractions for attempting the isolation of the individual substances. Bioactivity was reaffirmed (at less than 0.1 mg in 70 µL) in two subfractions, one which was able to decrease the primary readout from the FAS-HCS assay in a Caco-2 infection model, and another one which inhibited the growth of EPEC (III Figure 5). We then extracted the individual peaks for study and their elementwise composition was uncovered using HPLC/MS (see table 6). However, at this point we had exhausted the supply of the fractions, so we recultured these strains, performed the fractionation again and analyzed these individual constituents using MS and NMR. Judging by the mass spectrometry features, the actin pedestal inhibiting compounds appear to be large phospholipids with closely related structures, not resolved via the used HPLC methods. We do not know the mode of action of this effect, but EPEC are known to bind cell surface phospholipids, for example to phosphatidylethanolamine, using the bundle forming pilus (Khursigara et al. 2001). These phospholipids may be parts of the bacterial cells that were present in the culture media. The growth inhibiting compound may be a peptide, however, we were not able to uncover the structure of this compound in NMR experiments. We do not know the concentration of these compounds in the tested fractions but they are likely to be extremely low.

Table 6. Elemental Composition from ESI+ data.

Compound	observed *m/z* and observed RT	calcd. *m/z*	Mass error:	observed CCS	Formula:	final yield
1	782.46051 $[M + H]^+$ 7.01 min	782.46082	0.396186 ppm	279.99 $Å^2$	$C_{41}H_{68}NO_{11}P$	1.2 mg
2	796.47660 $[M + H]^+$ 7.39 min	796.47647	0.163219 ppm	281.52 $Å^2$	$C_{42}H_{70}NO_{11}P$	0.9 mg
3	316.10369 $[M + H]^+$ 3.7 min	316.10458	2.815524	160.04 $Å^2$	$C_{14}H_{13}N_5O_4$	-

6 Concluding remarks and future perspectives

Drugs with virulence blocking properties could revolutionize the treatment of bacterial infections. This is because the current standard of care is based on compounds indiscriminately lethal to all bacteria, both commensal and pathogenic and rapidly drive resistance. In contrast, virulence-preventing treatments could barely shift the focus of the pathogens from their symptomatic site, to other ones where they could continue proliferating their genes without issue.

This thesis contributed to understanding how difficult it is to screen for virulence inhibitors. In our first publication we studied the most widely used *in vitro* EPEC bioassay, the FAS assay and we show that it can be fully automated, including dispensing of cells, infection with bacteria, image capture and image processing. In the future, algorithms and large language models should be designed to take a more active role in the decision making within experimental design and fully plan the analysis of the data.

In the second study we applied computational drug design methods to the x-ray crystallography cocrystal structure of Tir and intimin. We were able to show that there are no good quality binding sites or clefts in the structure if one were to use traditional methods. Screening compounds from our in-house library and multiple commercial sources did not produce compounds that would change the phenotype in a relevant way without toxicity.

In addition, we cultured marine microbes, extracted their culture medium and searched for EPEC virulence inhibiting compounds from these. Most of the fractions did not display any kind of activity. Two fractions did display a minor activity, one inhibitory to growth and the other possibly preventing adhesion via an unknown mechanism.

In the future, better methods for the study of molecular interactions could be used as a starting point for the discovery of Tir-intimin inhibitors. In sum, this thesis contributes to the progress of drug discovery endeavors aimed at combating bacterial virulence factors. The discoveries outlined herein set the stage for future inquiries into drug development targeting Tir and underscore the constrained efficacy of conventional virtual screening and phenotype-based high-throughput screening approaches in expediting the identification of antivirulence compounds.

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Development and validation of a high-content screening assay for inhibitors of enteropathogenic *E. coli* adhesion

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ABSTRACT

Enteropathogenic *E. coli* (EPEC) causes intestinal infections leading to severe diarrhea. EPEC attaches to the host cell causing lesions to the intestinal epithelium coupled with the effacement of microvilli. In the process, actin accumulates into a pedestal-like structure under bacterial microcolonies. We designed an automated fluorescence microscopy-based screening method for discovering compounds capable of inhibiting EPEC adhesion and virulence using aurodox, a type three secretion system (T3SS) inhibitor, as a positive control. The screening assay employs an EPEC strain (2348/69) expressing a fluorescent protein and actin staining for monitoring the bacteria and their pedestals respectively, analyzing these with a custom image analysis pipeline. The assay allows for the discovery of compounds capable of preventing the formation of pathogenic actin rearrangements. These compounds may be interfering with virulence-related molecular pathways relevant for developing antivirulence leads.

1. Introduction

Gastrointestinal infections and diarrheal diseases are pervasive global health burdens, especially with ever-increasing levels of antimicrobial drug resistance (Fagundes-Neto, 2013). One considerable cause of diarrhea and intestinal infections is enteropathogenic *Escherichia coli* (EPEC), which exhibits resistance to a large range of antibiotics (Rodrigues, 2019; Eltai et al., 2020). Treating resistant gastrointestinal infections with antibiotics is particularly problematic because antibiotics also target protective normal flora, while possibly not eliminating resistant pathogens. As a consequence, an extensive amount of research has been put forward to develop so-called antivirulence drugs, that is, drugs capable of attenuating virulence, without eliminating bacteria (see Dickey et al., 2017 for review). Approved antivirulence drugs to date are compounds attenuating the effects of toxins, but preclinical research has also focused at other possible targets, such as various attachment adhesins and appendices, cell-to-cell communication (quorum sensing), inhibition of specialized bacterial secretory systems, and virulence gene expression (Buroni and Chiarelli, 2020; Theuretzbacher et al., 2020).

The virulence of EPEC, and many other human enteric pathogens is brought about by manipulating pathways of membrane trafficking, cell death/survival and the modulation of the cytoskeleton, ultimately altering the macroscopic properties of the epithelium and provoking gastroenteritis (Mattoo et al., 2007). So-called effector proteins (virulence factors) are used by EPEC and most Gram-negative pathogens to achieve these effects. Effector proteins are injected into the host cell using the type three secretion system (T3SS), a needle-like injection appendage and associated effectors (Gophna et al., 2003). The effectors are typically carried through the needle by chaperones to the cytosol where they play various roles, often aiding the pathogen in invading the host and surviving host defense mechanisms. The size of the ensemble of secreted effectors varies between pathogens, from the four of *Pseudomonas aeruginosa* to several hundred in other pathogens (Lee et al., 2005). Due to the near universality of the T3SS, research has targeted this system in attempts to discover drugs capable of attenuating virulence without antibiotic mechanisms of action (Baron, 2010). The most advanced demonstration of the therapeutic usefulness of T3SS inhibition has been a phase 2 trial of an antibody for *Pseudomonas aeruginosa* infections by KaloBios Pharmaceuticals (Jain et al., 2018).

In typical presentations of EPEC infection, the bacterium adheres to intestinal epithelium, causing the localized effacement of microvilli, the

Abbreviations: A/E, attaching and effacing; EPEC, enteropathogenic *Escherichia coli*; LEE, locus of enterocyte effacement; T3SS, type III secretion system; HTS, high-throughput screening..

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abnormal polymerization of actin in its vicinity, and increasing the permeability of the epithelium (Moon et al., 1983). Initially EPEC adheres to cells using multiple appendices (e.g. pili) forming localized bacterial aggregates or microcolonies. This phenotype was named localized adherence by Scaletsky and colleagues (Scaletsky et al., 1984). After the initial stage of adherence, EPEC injects virulence molecules with the T3SS into the host causing the bacteria to adhere more vigorously and initiate the formation of A/E lesions (Moon et al., 1983). The damage leads to the retention of water and the malabsorption of nutrients and therefore to diarrhea and other symptoms. While the time frame of the onset of symptoms suggests that changes in chloride ion secretion are the *de facto* main cause of acute diarrhea (Lapointe et al., 2009), A/E lesions and the associated actin pedestals are considered to be important facets of the illness allowing for chronic infections lasting up to 30 days (Fagundes-Neto and Scaletsky, 2000).

Our assay focuses on the fact that EPEC effectors manipulate the cytoskeleton, inducing so-called actin pedestals, protrusions of abnormal actin polymerization. While the exact function of actin pedestals is unknown, they may be a form of niche construction and function to protect the pathogen from environmental stress, for example shear within the intestines, and host immune responses such as phagocytosis (for discussion on theories of possible function see, for example Battle et al., 2014; Brady, 2007; Cepeda-Molero et al., 2020). Compounds or structural motifs capable of altering the actin polymerization phenotypes caused by bacterial pathogens, could be used as starting points for the development of antivirulence drugs especially since deletion mutants lacking some of the effectors involved tend to show either attenuated virulence or the lack of it entirely. For example, the deletion of EPEC *eae* – a gene encoding a C-lectin adhesin intimin, expressed on the bacterial outer membrane and responsible for binding to a translocated receptor, Tir – produces mutants incapable of forming pathogenic actin rearrangements and showing decreased adhesion in cell culture models (Cleary, 2004), although in cultured human biopsies other effectors are also required (Cepeda-Molero et al., 2017). Similarly, the deletion of *espA,* a gene encoding one of the main structural proteins of the T3SS appendage, produce mutants showing decreased adhesion to cells (Cleary, 2004).

To our knowledge, high-throughput assays monitoring pathogen-induced actin rearrangements have not been reported before. We therefore designed a high-content fluorescence-based imaging assay using the previously described fluorescent actin staining assay (Knutton et al., 1989), a fluorescent EPEC strain, and readily available license-free image analysis software. This assay is capable of assessing the capacity of compounds to inhibit A/E lesion-associated actin rearrangement.

2. Materials and methods

2.1. Assay design

The overall workflow of the assay is shown in Fig. 1. The assay in its entirety can be run in two days, where the first day is required for preparation of cell and bacterial cultures. On the second day the infection, staining, and imaging are done. After the plates are fixed, they can be stored without considerable degradation for at least a week in the dark at +4 °C until imaging is performed.

2.1.1. Creation and validation of the fluorescent bacterial strain

Escherichia coli 2348/69, a typical EPEC type 1 strain, was obtained from Reference Microbiology Services, UK. Liquid overnight cultures were grown in LB broth at 37 °C and 200 rpm from single colonies taken from a weekly renewed LB agar culture. For image acquisition, the strain was transformed with a plasmid to constitutively express fluorescent protein mCherry. To achieve this, competent bacteria were made using the rubidium chloride method (Bird, 2011). The plasmid pON.mCherry, Addgene #84821 deposited by Howard Schuman (Gebhardt et al., 2017), was extracted using a miniprep kit (GeneJET, Thermo Fisher Scientific K0502). After this, the extracted plasmid was incubated with the competent bacteria and the transformants were selected with 30 μg mL^{-1} chloramphenicol and deposited into long-term storage (−80 °C) as glycerol stocks.

To assess stability of the fluorescent strain, we proliferated and incubated the transformed bacteria in conditions identical to those used in the assay. After pre-incubation in MEM, 100 μL suspensions (2×10^6 cfu mL^{-1}) of bacteria were placed into an empty imaging 96-well plate

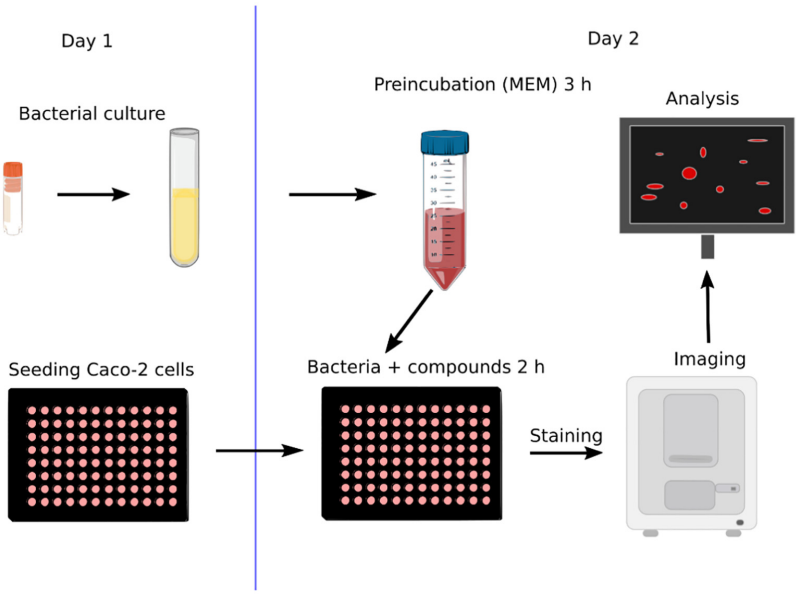


Fig. 1. The assay workflow. On the first day, Caco-2 cells are seeded to a well plate while an overnight liquid culture is initiated from the glycerol stock of the bacteria. The following day, bacteria are first preincubated in serum-free MEM for three hours and used to infect the cells. The cells are then incubated with the bacteria for two hours and exposed to test compounds or the aurodox control. The infection is terminated by adding the fixative-containing staining solution, after which the plate is imaged, and the images processed.

(Viewplate, Perkin Elmer) where they were imaged with phase contrast and fluorescence microscopy at 20×. The resulting images were processed principally following the ideas described by Canny (1986) and enumerating the individual bacteria in both the phase contrast and the fluorescence image to estimate the proportion of fluorescent bacteria in the population.

To demonstrate that the transformed EPEC strain pertained pathogenicity, we infected the cells with the transformed strain and the wild type side-by-side using antibodies to visualize the non-fluorescent bacteria. Cells infected with non-fluorescent wild type bacteria were first washed three times with Hank's Balanced Salt solution (HBSS), and fixed in 4% paraformaldehyde for ten minutes. The cells were then washed thrice with PBS and blocked using 1% bovine serum albumin (Merck) for one hour. After this, the cells were permeabilized with 0.1% (*v*/v) Triton X-100 and 1% (*w*/*v*) BSA in PBS for three minutes and washed thrice with PBS. Next, the samples were incubated with *E. coli* serotype O/K polyclonal antibody (PA1-73032, Thermo Fisher Scientific) 1:100 and 1% BSA for two hours. The cells were washed thrice with PBS, then incubated with Rabbit anti-goat IgG (H + L) Cross-Adsorbed antibody conjugated to AlexaFluor-594 (Thermo Fisher Scientific) 1:1000 and 1% BSA, for one hour, and washed with PBS thrice. For the analysis of F-actin, AlexaFluor-488-conjugated phalloidin (Thermo Fisher Scientific) was applied (20 pM) for 20 min and for nuclei Hoechst 333432 (Merck) (3 μg/mL) for three minutes. Then cells were washed three times with PBS before imaging. All these steps were performed at room temperature.

2.1.2. Infection of mammalian cells

Human epithelial colorectal adenocarcinoma Caco-2 cells (ATCC HTB-37) were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% non-essential amino acids at 37 °C, 95% humidity and 5% CO_2. All cell culture reagents were obtained from Thermo Fisher Scientific.

The infection of Caco-2 cells was performed essentially as described previously (Cravioto et al., 1979; Knutton et al., 1989). The cells were seeded onto poly-D-lysine (Merck) coated, black, clear bottom 96-well plates (Viewplate, Perkin Elmer) at 4×10^4 cells per well and incubated for 24 h before the infection. Overnight bacterial liquid cultures were proliferated directly from the glycerol stocks, by cultivation in sterile glass tubes in LB broth supplemented with 30 μg mL^{-1} chloramphenicol at 37 °C, 200 rpm. Bacteria were then preincubated as described previously (Vuopio-Varkila and Schoolnik, 1991) in a nutrient-deficient medium (MEM without serum) to promote the expression of virulence factors including bundle forming pili (Vuopio-Varkila and Schoolnik, 1991) and locus of enterocyte effacement (LEE) gene products (Hazen et al., 2017). Liquid overnight cultures were diluted 1:50 in serum-free MEM and placed for three hours at 37 °C without shaking in a centrifuge tube with filter cap (Corning mini bioreactor 431,720). At this point, we also added the positive control, aurodox (1–10 μg mL^{-1}) (BioVision), into one of the bioreactors. Finally, the preincubated bacteria were added to the well plates at a multiplicity of infection (MOI) of 1:10 suspended in serum-free MEM. The plates were centrifuged (Eppendorf 5840R) for four min at 1000 ×g for the synchronization of the infection, and subsequently incubated for two hours at 37 °C and 5% CO_2.

2.1.3. Fluorescence staining

Cells infected with the mCherry-expressing EPEC were fixed and stained following a protocol published earlier (Knutton et al., 1989), with slight modifications to make it more amenable for high-throughput assays. An equivalent volume (100 μL) of staining solution [8% paraformaldehyde, 0.2% Triton-X (Thermo Fisher Scientific), 40 pM phalloidin-AlexaFluor-488 (Thermo Fisher Scientific), 6 μg/mL Hoechst 33342 (Thermo Fisher Scientific) in PBS] was added directly to the incubation medium for 20 min. The cells were washed three times with HBSS before imaging.

2.1.4. Imaging

After the cells were infected and stained as described above, they were imaged using Cytation 5 (BioTek) with 20× Plan Fluorite phase contrast NA 0.45 objective (Olympus 1,320,517) and filter cubes for DAPI (1225100) to visualize Hoechst 33342, Texas red (1225102) to visualize mCherry and GFP (1225101) to visualize AlexaFluor-488. Focal plane stacks of 20 images with distance 0.8 μm of each other were taken from five non-overlapping fields of view within each well. The locations of these were the same for each well as the microscope does not allow imaging at random locations. A stack of focal planes and their projection was used to ensure capturing in-focus actin pedestal images over the entire field of view as the pedestals are not always entirely on the same z-plane. We used three of the images from the stack for the projection, the rest of the – mostly out-of-focus – stack images were excluded from the analysis. Taking such excessive image stacks is due to a limitation of the imager, which does not allow to take a focal plane stack of three images from different focal points for the three different channels separately. Data was reduced from all five fields of view and an arithmetic mean was calculated to represent the data for a particular well. More than one field allows for a better estimate of the well-level effect and also protects against failures in image acquisition as the probability of one image capture failing is substantially larger than that of five independent captures.

2.1.5. Image preprocessing and analysis

The raw images were preprocessed and analyzed using scikit-image 0.17 (van der Walt et al., 2014). The preprocessing stage consists of an algorithm which estimates the quality of the images before proceeding to the analysis. This includes the estimation of the degree to which cells occupy each field of view (confluency of the monolayer) and evaluates how well the autofocus algorithm of the microscope worked. Confluency is estimated by comparing the total image pixel count to the actin-stained areas that can display fluorescence signal, as this approaches 100% when there is an intact monolayer over the entire image. Bacteria bind to the plate giving misleading readouts if there are areas void of cells, since they cannot cause actin pedestals there. Similarly, out-of-focus images are detected by the "variance of the Laplacian"-method from the bacterial signal (International Conference on Pattern Recognition et al., 2000). Sometimes the autofocus algorithm of the imager would focus on a floating detached cell or debris or fail for no obvious reason. The captured image is convolved with a 10 × 10 Laplacian kernel to acquire the second derivative of the image and the variance of the transform is measured. Properly focused images generally have higher variance because of sharp contrasts, whereas images with issues in focus, such as blur, will have lower than expected values. Low quality images were then flagged and automatically removed, as they were rare events. Low quality was defined as either more than 10% of the image missing cells or a variance of the Laplacian more than three standard deviations from the mean. When an image was rejected in this manner, it meant that the well-level data was produced by averaging only four, and not five, fields of view for one well. In other words, no well level data was deleted or marked as an outlier as is often done in screening.

A set of features was extracted from the preprocessed images. The microcolony counts were extracted by first uncovering a threshold value via Otsu's method, which minimizes interclass intensity variance for signal and background (Otsu, 1979). Subsequently, this threshold value was used to create a binary (foreground/background) mask, which was then used for labeling all connected components from the bacterial signal with a Union Find algorithm and counting each object (Fiorio and Gustedt, 1996)(depicted in Fig. 2A).

To enumerate the pedestals, we applied the created binary mask to the image of the actin staining captured from the equivalent location to define regions of interest (Fig. 2B) and saved them as individual images (examples in Fig. 2C). These collections of segments from each field of view were then subjected to further analysis, where contours were

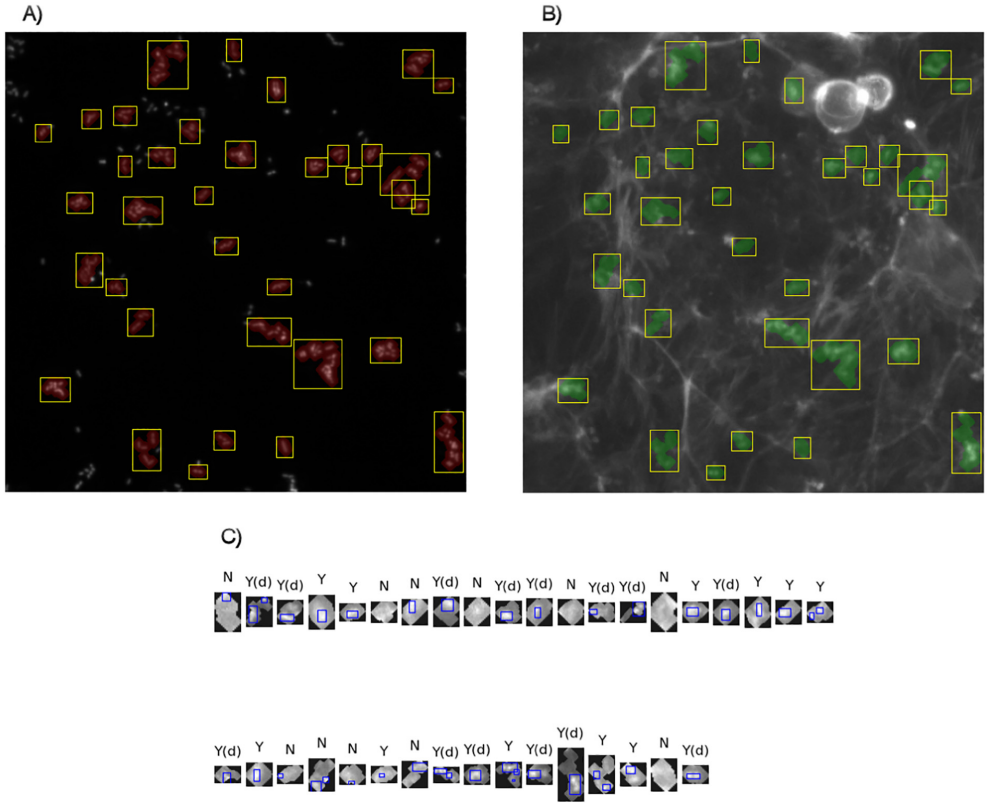


Fig. 2. Image processing methods. A) First, a mask is created from the fluorescence signal emitted by the bacteria (mCherry). The mask is shown in red and corresponds to the areas where the microcolonies are located. B) Then the equivalent regions are cut out from the actin signal in the primary segmentation step (phalloidin stained image). C) Lastly, secondary segmentation is performed within the collected primary segments, here marked with a blue bounding box. Images classified Y = pedestal, N = not, d = dense as described in section 2.1.5. These images are cropped for presentation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

searched for from within them by tracking the most intense pixels by another round of segmentation (Fig. 2C). This is based on the notion that actin pedestals are excessive polymerization of actin, which would lead to higher local concentrations of the fluorophores. If these secondary segments from within the primary segments were co-located with a microcolony – that is they also had higher than threshold fluorescence from the equivalent region in the bacterial signal – they were kept and counted as pedestals, otherwise they were discarded as artifacts or other actin condense structures not related to bacteria. After summing up all the colonies and pedestals in this way, they were combined into a descriptive proportion number $\hat{p}(x)$, by dividing the amount of pedestals (all segments, s_i that meet the conditions x for being a pedestal) by the amount of colonies (n):

$$\hat{p}(x) = \frac{1}{n}\sum_{i}^{n} |s_i = x|$$

In addition, we measured bulk fluorescence, microcolony size and density of the pedestals, estimated by computing the distance from the minimum to maximum intensity within the saved individual images (shown in Fig. 2C).

Plotting was performed using the graphing tool, GR with the StatsPlots library of Julia, and scientific color maps (Crameri, 2019). Statistics were calculated with libraries of Julia 1.6 (Bezanson et al., 2017).

2.1.6. Selection of the positive control

The assay protocol was executed as described above (see 2.1) while various candidate compounds were added in a range of concentrations (Table 1). Aurodox was administered in advance as explained above (see 2.1.2) while other substances were administered to the bacteria upon infecting the cells. For selection we used a random intercept and random slope linear mixed effects model to detect any monotonic concentration-dependent response and evaluate the intraclass correlation of the measurements within the experiments. As selection criteria we used the

Table 1
Compounds considered as positive controls for the assay.

Control compound	Concentrations	Proposed mechanism of action	Source
aurodox (Kimura et al., 2011)	0.1–10 μg mL^{-1}	down-regulation of LEE-genes	BioVision
lactoferrin human, recombinant, (rice), iron saturated, ≥90% (Ochoa et al., 2003)	5–20 mg mL^{-1}	multiple mechanisms; preventing needle complex	Merck
peptide (YFPYSHTSPRQP) (Li et al., 2016)	50–200 μg mL^{-1}	blocking of EspB	Merck
genistein	16–67 μg mL^{-1}	kinase inhibitor	Merck

model slope and between-experiment variability (Supplementary data Table S1).

2.2. Assay validation

2.2.1. DMSO tolerance

As library compounds are often stored and administered to assays in DMSO, an experiment was carried out to assess the effects of DMSO on assay readouts. The assay protocol was executed as described above (see 2.1). Various concentrations (1–10%) of DMSO were added into the bacterial suspension for the duration of the infection.

2.2.2. Aurodox tolerance

Because the positive control was reported to have antimicrobial effects (Kimura et al., 2011; Maehr et al., 1979), an experiment was carried out to assess its capacity to inhibit bacterial growth. Bacteria were grown overnight and preincubated as described in 2.1.2. A suspension of about 4×10^6 bacteria /mL was prepared into fresh LB broth with chloramphenicol, dispensed into a white 96-well plate (100 μl/well) and exposed to different concentrations (2.5–10 μg mL^{-1}) of aurodox, to LB as a non-treated control and to 70 μg mL^{-1} streptomycin sulfate. After two hours, bacterial titer was assessed with an ATP-based kit BacTiter-Glo (Promega) measuring luminescence for 1000 ms on Varioskan LUX (Thermo Fisher Scientific).

2.2.3. Assay reproducibility

To evaluate the reproducibility of the assay between distinct days and variation between the wells within one individual plate, we followed the Assay Guidance Manual (Iversen et al., 2004). The assay was performed on three separate days by plating a maximal concentration (5.0 μg mL^{-1}) of the positive control aurodox, a lower concentration (2.5 μg mL^{-1}), and non-treated control at varying locations on the plate. After incubation, these plates were stained, imaged and analyzed as described above (see 2.1). We used Z' and Signal Window (SW) as effect size and variance metrics to assess the responses of the screening assay:

$$Z' = 1 - \frac{\left(\widehat{\sigma}_{max} + \widehat{\sigma}_{min}\right)}{\left|\widehat{\mu}_{max} + \widehat{\mu}_{min}\right|}$$

$$SW = \frac{\widehat{\mu}_{max} - \widehat{\mu}_{min} - 3\left(\widehat{\sigma}_{max} + \widehat{\sigma}_{min}\right)}{\widehat{\sigma}_{max}}$$

where μ_{max} is the arithmetic mean of the high value sample (no inhibition) and μ_{min} is the low value sample mean, σ is the sample standard deviation.

3. Results and discussion

In past years, traditional target-based high-throughput screening (HTS) for antimicrobials has been essentially unsuccessful. An analysis of 70 HTS campaigns conducted towards essential genes of bacteria at GlaxoSmithKline during 1995–2001 described an end result of no workable drug candidates (Payne et al., 2007). A similar later analysis (2001−2010) of 65 HTS campaigns by AstraZeneca also failed to produce any (Tommasi et al. 2015). The authors of the later paper opinion that among other things, such campaigns utilize very reductionist assays uncovering active structures either not being capable of entering bacteria or being effluxed by membrane pumps.

Issues such as this have led to increased interest in whole-cell assays where the critical capacity of compounds to reach the target is assessed already during screening. For example, in the absence of any known shared molecule to target with ligand-based design, multiple whole-cell screening assays have analyzed phenotypic changes to uncover small molecule T3SS-inhibitors, leading to many publications on the structure-activity relationships and chemical optimizations of analogic structures (Gu et al., 2015). Among these are, for example, salicylaldehyde hydrazones and analogs, which are capable of inhibiting T3SSs in multiple pathogenic species of *Yersinia*, multiple *Chlamydias*, *Salmonella* and EPEC (Kauppi et al., 2003; Gauthier et al., 2005; Negrea et al., 2007). Independently of each other, Kauppi et al. (2003) and Gauthier et al. (2005) discovered similar chemical structures capable of inhibiting T3SS in *Yersinia pseudotuberculosis* and EPEC. Kauppi et al. (2003) used a whole-cell reporter gene assay to screen 9400 compounds against *Yersinia pseudotuberculosis* T3SS and Gauthier et al. monitored EspB secretion from the T3SS of EPEC in culture using ELISA against 20,000 Maybridge compounds. Similarly, Pan et al. (2007) used an avirulent but luminant *Yersinia pestis* to screen 70,966 compounds, finding previously recognized compounds but also other ones with entirely new structures. Yet another similar high-throughput approach is the absorbance-based monitoring of T3SS-dependant red blood cells lysis first described by Warawa et al. (Warawa, Finlay, and Kenny 1999). Screening using this method has led to the rediscovery of the antibiotics aurodox and factumycin as down-regulators of the T3SS (Kimura et al., 2011).

In a similar fashion, our primary objective was to create a high-content screening assay for EPEC pedestal formation using a whole-cell infection model. Therefore, we designed an automated fluorescence-imaging assay in which cell monolayers are infected with bacteria, and an infection phenotype is monitored using actin binding fluorophores. This assay is an automated, 96-well plate adaptation of the EPEC-Caco-2 adherence and fluorescence microscopy assay described by Knutton et al. (1989) supplemented with a suitable positive control and data reduction method based on extracting actin pedestals from the images automatically in high-throughput.

3.1. Assay design and validation

Expressing foreign proteins – such as the fluorescent protein we used – in pathogens, can change important biological properties of the microorganism, in the worst case rendering the entire assay invalid. Therefore, we evaluated whether the transformed strain created for the assay can cause comparable actin rearrangements on the cells leading to similar assay readouts. For this purpose, cells were infected with both the wild type EPEC strain, and the transformed fluorescent strain and compared. Both strains could adhere to the Caco-2 monolayer in microcolonies, infect the cultured cells, and produce similar actin polymerization phenotypes and readouts (Supplementary data Figs. S1-S2). We also carried out an experiment to evaluate the stability of the fluorescent strain in the assay (Supplementary data Figs. S3-S5). The results show that on average about 93.7% ($CI_{95\%}$ 89.3% - 96.7%) of the bacteria can be perceived with fluorescence imaging. A small proportion of non-fluorescent bacteria may be present in the assay, although non-viable bacteria are lost in washing steps of the assay. However, our methodology only uses the fluorescence signal to create a mask for downstream processing and this does not require every single bacterium to be fluorescent.

Since several substances have been reported in the literature to be capable of preventing EPEC A/*E*-lesions in *in vitro* models, we studied if they could also prevent actin pedestals in our assay and be used as controls. Most of these substances work by perturbing the T3SS in some manner (see Table 1).

Recombinant human lactoferrin did not show any effect on pedestal formation ($\beta = -0.001$, $p = 0.7$, see Fig. 3) according to our results. Ochoa et al. reported that lactoferrin prevents actin pedestals "due to interference with the initial step in EPEC mammalian cell attachment" (Ochoa et al., 2003). This discrepancy may be explained by the fact that lactoferrin does not decrease the proportion of colonies with pedestals but does decrease the absolute counts of actin pedestals by preventing the initial attachment to cell monolayers. Ochoa et al. analyzed actin pedestals in HEp2 monolayers by counting the HEp2 cells as positive for actin pedestals if they had microcolonies of at least ten bacteria and

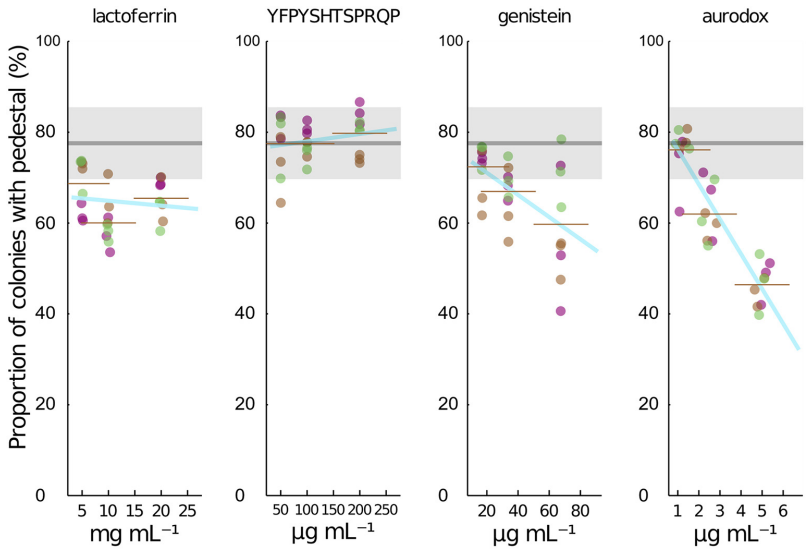


Fig. 3. The effects of positive control candidates on the proportion of colonies with pedestals in the assay. The peptide (YFPYSHTSPRQP) did not show any dose-dependent effect, lactoferrin had no effect on the readout, except that the intercept was slightly lower, due to it inhibiting normal colony formation. Genistein and aurodox displayed a dose-dependent decrease in the assay readout, though genistein demonstrated large between experiment variation. Data points represent means of five fields of view from three individual wells from three independent experiments performed on distinct days. The non-treated control (MEM only) is indicated in each panel as a grey line and a ribbon indicates the 95% confidence interval of the mean.

associated actin pedestals as evaluated by eye. They do not therefore address the issue of whether the microcolonies present display lesser relative degree of pedestal formation or not.

We noticed, additionally, that lactoferrin at 5.0 μg mL^{-1} did not decrease the total amount of bacteria present in the images, but rather it increased microcolony counts (230% compared to the non-treated control, Supplementary data Fig. S6) while decreasing microcolony average areas by 36% (Supplementary data Fig. S6). In contrast, compounds purely detrimental to microcolony formation – such as 10% DMSO – similarly decrease average sizes, but they also decrease total microcolony counts (Supplementary data Fig. S6). Interestingly microcolony sizes display a very uneven Pareto or exponential degree distribution, where the large majority have small sizes while a few colonies have large sizes. Indeed, this is what one would expect if bacterial aggregation to colonies displays preferential attachment and can be modelled with the Barabási–Albert model as most biological network phenomena can (Barabási, 2009; Grinberg et al., 2019). In other words, a new adhering or forming bacterium is likelier to appear in a colony with more members than into a small one. In any case, this also means that counting colonies above a certain size – such as that in the work of Ochoa et al. – will exclude more of the microcolonies in the lactoferrin condition than in other conditions not affecting the size. Thus, it is possible to see an increase in total microcolony counts, while simultaneously seeing a decrease in cells positive for actin pedestals. Ochoa et al. also used a four-hour incubation time and much higher MOI (1:500) than used in this work. Due to lactoferrin's capacity to change the localized adherence phenotype, the assay we describe here can be used to probe the effects of library compounds on microcolony formation using 5.0 mg mL^{-1} lactoferrin as a control.

A peptide (YFPYSHTSPRQP) reported to be capable of preventing localized adherence – an early attachment phenotype of some EPEC strains (Li et al., 2016) – resulted in no measurable decrease in the number of actin pedestals ($\beta = 0.0002$, $p = 0.19$, see Fig. 3). Neither did it have any effect on EPEC microcolony size nor counts (Supplementary data Fig. S6). Li et al. used a much shorter infection time (30 min) and did not analyze pedestals, but only adhesion.

Genistein, a kinase inhibitor used as a control in many assays involving actin rearranging pathogens (Coconnier et al., 1998; Rosenshine et al., 1996) was capable of preventing actin pedestals in our assay ($\beta = -0.002$ $p = 0.04$, see Fig. 3). There was, however, considerable variation between experiments, which perhaps are not due to properties inherent to genistein, but rather further optimization would be needed. The duration between pipetting the suspensions and the plate reaching the incubator likely needs to be very precise, since the effect of genistein is known to be enhanced by short pretreatment of the cells before infection. However, as genistein is a promiscuous kinase blocker with multiple other inhibiting effects on normal cell signaling, it also interferes with the normal biology of the host cell to some degree.

Aurodox – an antibiotic produced by *Streptomyces goldniensis* (Maehr et al., 1973, 1979) – is a member of the elfamycin family of antibiotics first described in the 1970's (Maehr et al., 1973). It was rediscovered as a T3SS inhibitor in 2011, in a screening campaign utilizing natural product libraries (Kimura et al., 2011). The exact mechanism of action of aurodox is not entirely understood, but like other antimicrobial substances extracted from *Streptomyces*, it inhibits protein biosynthesis, although through an elongation factor (Tu), and not by directly inhibiting ribosome activity (Parmeggiani and Swart, 1985; Vogeley et al., 2001). This down-regulates the transcription of T3SS-related genes in the locus of enterocyte effacement (LEE) via the repression of a master regulator (*ler*) (Elliott et al., 2000). These genes are known to be necessary for the induction of actin pedestals in *in vitro* organ culture (Cepeda-Molero et al., 2017). In our assay we pretreated bacteria with aurodox due to its translation-mediated mechanism of action. Aurodox both decreased the quantity of perceivable pedestals ($\beta = -0.076$, $p < 0.001$, see Fig. 3) and their density suggesting that it partially prevented actin condensation underneath the colonies. Aurodox at concentration of 5.0 μg mL^{-1} was used in earlier high-throughput assays (Kimura et al., 2011) and it only slightly inhibited the growth of the transformed bacteria (Supplementary data Fig. S7). We therefore chose aurodox at 5.0 μg mL^{-1} concentration as positive control for further assay validation steps.

Most screening library compounds are stored and delivered in DMSO. The ability of Caco-2 cells to tolerate DMSO concentrations up to 10% without substantial membrane damage or changes in apical membrane permeability has been reported in the literature (Da Violante et al., 2002). Therefore, we conducted an experiment to assess the tolerance of the assay to DMSO at 1–10% of the total volume. DMSO concentration did not significantly alter the readout, but at

concentrations above 5% cell detachment increased lowering the values and causing increased variation (Supplementary data Fig. S8). However, total fluorescence emitted by bacteria decreased in a concentration-dependent manner (Supplementary data Fig. S9) suggesting possible toxicity or degradation of the fluorescent protein. Thus, total DMSO concentrations in this assay should not exceed 5% of total well volume.

In order to assess assay stability over different plates and days, we ran nine assay plates – three separate plates on three separate days – and analyzed the data following procedure and the acceptance criteria described in the assay guidance manual (Iversen et al., 2004). The results show a consistent signal over different plate regions (Fig. 4). There were no considerable between-plates differences, nor any difference over the distinct days; all within-day and between-day fold shifts were below two. There was also low variance between wells, the intraplate coefficients of variance being under 0.2 for the primary readout on average, while the signal windows (SW) were above 2 for all plates.

We calculated the Z'-factors on the pedestal counts as this data is on a scale that is more suitable for Z' than a bound proportion of counts (Iversen et al., 2006; Sui and Wu, 2007). Z' was designed for traditional HTS readouts and is not so well suited for imaging assays (Iversen et al., 2004). On average our assay scored a Z'-factor of 0.44 with four production assay replicates and has a $\sigma_{max}/\sigma_{min}$ of 1.0. This is equivalent to a $T_{80\%} \geq {}_{50}$ of 56.1, meaning that at a cut-off for hit selection of 56% inhibition this assay will recognize 76.7% of all compounds truly inhibiting at more than 50%, while only 20% of the compounds identified as active will not truly inhibit by more than 50% (Bar and Zweifach, 2020). However, the level of required replicates can be decreased by either using higher concentration of aurodox as a control or increasing the cut-off level to proclaim a compound active.

In order to be able to automatically screen the images, we designed an image analysis pipeline written in Python, in which we counted the amounts of pedestals in the field of view. Because Caco-2 cells display congenital structures rich in actin, processing fluorescent actin staining images via mere segmentation is not optimal for this complex assay set-up. Since the relevant actin rich pedestal structures are located underneath adherent bacteria, we processed the images by using the bacterial signal as a mask on the actin image. This process focuses the analysis on a very small part of the entire image (a region of interest, depicted in Fig. 2C), thus making it more specific and less prone to include false positives compared to simple segmentation from the actin channel, while remaining computationally light. At times the regions of interest may contain other actin rich structures not related to bacteria, or that are present over the entire segment with higher signal than that caused by the pedestal. To deal with this issue, we added a third step where the captured segments were validated by allowing only those with concurrent mCherry signal. This for the most part avoids capturing actin rich areas that are not related to bacteria and allows to achieve an adequate assay signal window.

Additionally, the total fluorescence intensity signal window from minimum to maximum, from the actin staining was measured as an indicator of the density of the actin condensation at that region. This was done because our control substances were capable of not only decreasing the count of pedestals, but also their total density, and such a number gives a means to estimate qualitative degradation of the pedestal structures in a production screen, in addition to simply enumerating them. This is particularly important, as there might exist library compounds that interfere with the condensation in multiple distinct ways.

Data extracted from the multiple fields of view were then aggregated by calculating their arithmetic mean. We tested the effect of using multiple fields of view on both the error of the estimate and on the variation between wells, by calculating the standard error of the mean and assay variability related parameters from data derived from one field of view, then adding additional fields of view until the results converge. As expected, increasing the number of fields of view used for the estimate decreases the level of error with diminishing returns (Table 2). The average standard error of the mean for the main readout

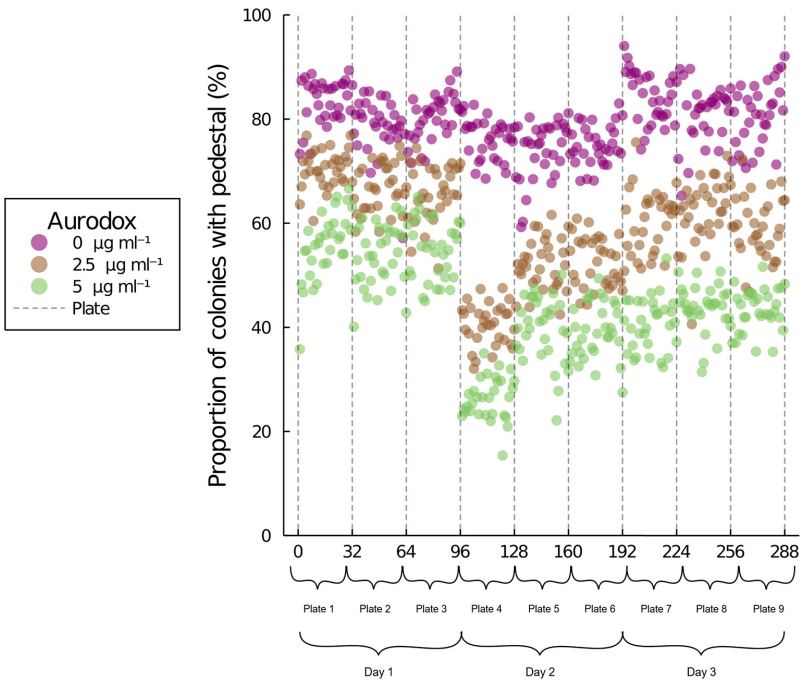


Fig. 4. Assay reproducibility over distinct plates and on various days. The plot shows results from three full 96-well plates on three distinct days (total of nine plates). The assay was performed by dispensing a maximal concentration (5.0 µg mL^{-1}) of the positive control aurodox, a lower concentration (2.5 µg mL^{-1}), and non-treated control (all with $8 \times 4 = 32$ wells) at varying locations on the plate, following guidelines of the Assay Guidance Manual (Sittampalam et al., 2004). Each data point represents an average measurement of five fields of view from one well.

Table 2
The number of fields of view decreases the error of the well level readout estimate, while increasing the signal window (SW) and Z' factor of the assay. These improvements are increasingly smaller in magnitude, however.

Number fields of view	SEM for well-level data	SW of plate	Z' of plate
Two	3.8	3.28	0.40
Three	3.0	4.82	0.48
Four	2.6	4.95	0.49
Five	2.5	4.35	0.47

decreased from 3.8 to 2.5 when moving from a mean of two fields of view to one of five, but the increment was smaller after each addition to the point that the last addition barely showed improvement (Supplementary data Fig. S10). The improved well-level estimate then decreased the between-well variation in such a manner that the SW and Z'-factor increased from 3.28 and 0.4 to 4.35 and 0.47, respectively. With five fields of view, the within-well coefficients of variation (CV) for the main readout were below 0.2 (Supplementary data Fig. S11).

3.2. Assay application areas

Above we discussed other whole-cell screening methods used to screen for T3SS inhibitors. While these do use whole-cell setups and wild type strains, they either do not model an actual infection of mammalian cells or analyze fairly crude endpoints, such as cell death or lysis. Our method is based on the monitoring of the phenotypic changes in the actin cytoskeleton, and therefore allows following the pathological changes to actin polymerization, in particular, in the case of some EPEC strains and their associated actin pedestals. In contrast to target-based assays, this phenotypic assay potentially allows to identify compounds interfering with a variety of target molecules involved in eliciting actin pedestals. In addition, pathogens may cause abnormal actin polymerization recognizable in images but nevertheless be incapable of red blood cell lysis or other effector-based damage to cells. Additionally, this assay can be used to monitor the earlier stages of EPEC adherence via following colony morphology and could be used to screen inhibitors of localized adherence and autoaggregation (Hyland et al., 2006). Obviously, however, imaging and the respective data analysis require specialized equipment and is more laborious. A limitation of our approach is that not all clinical EPEC isolates show actin polymerization in this assay *in vitro* (Knutton et al., 2001; Knutton et al., 1991). Such strains may nevertheless be pathogenic and cause A/E lesions *in vivo* (Rocha et al., 2011). One challenge of designing the assay was the selection of a positive control substance. Many substances are used in the literature for assays dealing with actin modulating pathogens or, alternatively, are known to prevent effector injection via the T3SS. Not all of them, however, are readily available or capable of creating a robust dose-dependent response via specific, non-lethal, effects preferably on the bacteria alone. While aurodox certainly can prevent actin pedestals, the bacteria need to be pretreated before-hand due to the translation-mediated mechanism of action. In most screening settings this is not, however, something that can be reasonably done when screening extensive numbers of compounds, and one is then restricted to searching for compounds of more rapid manners of inhibition.

Given that multiple pathogens cause A/E lesions or use other cytoskeletal manipulation, it might be possible to extend the assay described here to study these other pathogens (Stradal and Schelhaas, 2018). Pathogens capable of causing the A/*E*-lesion phenotype and associated actin pedestals include enteropathogenic *Escherichia coli*, enterohemorrhagic *Escherichia coli* (EHEC), *Escherichia albertii*, a murine specific strain *Citrobacter rodentium*, and rabbit enteropathogenic *Escherichia coli* (Yamamoto et al., 2017). EHEC strains, for example, also cause pedestals in cultured cells, although according to Knutton et al., they do not all attach to Caco-2 cells. The authors, however, also reported that EHEC strains of serotypes 0157:H7 and 026:H11 do adhere to Hep-2 and HEL cell monolayers, suggesting that these might be reasonable alternative host cells (Knutton et al., 1989). Various other abnormal actin accumulation phenotypes are described in the literature (Stevens et al., 2006). For example, "comet tails" created by *Listeria*, *Rickettsia*, *Bulkholderia*, (Ambrosi et al., 2012; Jasnin et al., 2013; Valvano et al., 2005) "ruffles" by *Salmonella* (Francis et al., 1993), and expanding the image analysis and classification algorithms to distinguish these phenotypes is possible. While aurodox, which is used here as a positive control, may not be suitable for use with other pathogens, salicylaldehyde hydrazones (Negrea et al., 2007) or actin polymerization disruptors, like cytochalasin D (Sanger et al., 1996) or tyrosine kinase inhibitors (staurosporine, genistein)(Benjamin et al., 1995), and possibly many more substances, can be substituted if needed.

3.3. Conclusions

Here we report the optimization and validation of HTS-compatible high-content imaging assay for screening for compounds inhibiting pathogen-induced actin pedestals. The method is based on a fluorescent EPEC strain accompanied by actin staining and therefore can be used for detecting molecules targeting effectors inducing actin pedestals in EPEC infection. With some modifications, this assay could be amended for studying other actin-modulating pathogens. The assay can be performed in 96-well format therefore enhancing the discovery of new anti-virulence compounds.

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Declaration of Competing interest

Authors declare no competing interest.

Appendix A. Supplementary data

The code used for analysis publically available athttps://github.com/tpylkko/FAS-HCS/. Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106201.

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Virtual Screening Assisted Search for Inhibitors of the Translocated Intimin Receptor of Enteropathogenic *Escherichia Coli*

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This study aimed to identify inhibitors of the translocated intimin receptor (Tir) of enteropathogenic *Escherichia coli* (EPEC). EPEC is an intestinal pathogen that causes diarrhea and is a major health concern worldwide. Because Tir is a key virulence factor involved in EPEC pathogenesis, inhibiting its function is a potential strategy for controlling EPEC infections. Virtual screening was applied to chemical libraries to search for compounds that inhibit Tir-mediated bacterial adherence to host cells. Three sites were targeted using the cocrystal structure published earlier. A selection of compounds was then assessed in a cell-based infection model and fluorescence microscopy assay. The results of this study provide a basis for further optimization and testing of Tir inhibitors as potential therapeutic agents for EPEC infections.

Introduction

It is widely accepted that the current collection of antimicrobial therapies is insufficient to counter the effects of pathogenic bacteria in the future. Classical antibiotic strategies have proven to be extremely successful and have transformed the world we live in.[1] However, they have drawbacks, for example, their non-specific nature causes unwanted effects by eliminating health-promoting bacteria. Furthermore, pathogens have managed to evolve many mechanisms of resistance, and these severely hinder the usefulness of such drugs. In fact, it has been shown that resistance to new types of antibiotics can be detected within a few years from their deployment in treatment.[2] One way to solve this problem would be by developing drugs capable of specific inhibition of pathogenic physiological reactions, without decreasing the viability of the pathogens. While such drugs would not be entirely immune to resistance-driving forces, the selection pressure would likely be much lower as the targeted pathogens could survive by switching to alternative lifestyles.

In practice, this could be achieved by pharmaceutical intervention directed towards virulence inducing molecules utilized by pathogens.[3] Most gram-negative pathogens use injected virulence molecules to infect host cells. Enteropathogenic *Escherichia coli* (EPEC) belongs to a subclass of such pathogens, often called attachment and effacing (A/E) pathogens.[4] These pathogens cause the effacement of intestinal microvilli and characteristic actin rich protrusions called pedestals, upon which EPEC stands on the plasma membrane.[4] This virulence cascade is not exclusive to EPEC; a highly similar process occurs in other A/E pathogens such as Enterohemorrhagic *Escherichia coli* (EHEC) and the mouse specific pathogen *Citrobacter rodentium*. These pedestals are known to be caused by virulence molecules transferred from the periplasm to the cytoplasm of the host cell via the type three secretion system (T3SS), a needle-like appendix that employs a molecular pump to do so.[4] Interestingly, the T3SS and the mentioned virulence molecules are encoded on a conserved pathogenicity island known as the locus of enterocyte effacement, prophages, or other insertion elements, meaning that many variations of the theme exist among A/E pathogens in the wild.

Among the proteins injected into the host's cytosol is a receptor known as the translocated intimin receptor (Tir).[4] This receptor is responsible for the aberrant actin condensation associated with the lesions caused by A/E pathogens. Tir integrates into the plasma membrane of the host cell, and works as a receptor for intimin, a ligand protein that is autotransported from the periplasm.[4] The structures of the translocated intimin receptor bound to intimin have been described using x-ray diffraction crystallography, shedding light on how the Tir interacts with intimin.[5] Ultimately, upon activation by the ligand, the receptor clusters[6] and interacts with downstream host adaptors and kinases responsible for actin nucleation, leading to the emergence of the pedestals and the destruction of microvilli.

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Interfering with this process could attenuate the virulence of EPEC, given that experiments have shown that Tir-intimin interaction is necessary but not sufficient to display pathogenic-like phenotypes in cell culture models and *ex vivo* transplants.[7] However, no orthosteric small molecule inhibitors of Tir have been found to date, despite the extensive research on the binding interaction. Ross and Miller demonstrated the existence of a binding hotspot consisting of three amino acid residues at the interface between Tir and intimin.[7] They then created short linear peptides containing these central Tir residues and tested their ability to bind the extracellular domain of intimin, but these did not demonstrate binding activity. This suggests that linear peptide mimetic inhibitors are insufficient for binding intimin, and other structural properties perhaps provided by buried residues are required for efficient binding. The authors speculate that this may be the structural preorganization of the β-hairpin residues of the receptor. The design of Tir binding antibodies and nanobodies has also been described (CA2479270C).[8] However, the nanobody did not bind to EPEC Tir. Additionally, as orally administered treatments, these would need to be able to resist digestion in the human gastro-intestinal tract.

We hypothesized that small molecule inhibitors of the Tir-intimin interface would effectively limit EPEC-induced lesions and thus virulence without compromising EPEC viability. Such molecules could possibly be administered orally avoiding many of the typical problem related to the development of orally administered pharmaceutical, such as reaching a high plasma and tissue concentration and avoiding efflux from the bacteria. Furthermore, since the sequences of EHEC Tir and EPEC Tir hairpin share 85% identity, in the best case, inhibitors of this target could also apply to other A/E pathogens.[9]

To answer this research question and discover such compounds, we applied virtual screening methods to three putative binding sites to select compounds from compound libraries and studied the compounds by using an actin pedestal detecting imaging assay.

Our aim was to provide insight into how this receptor could be inhibited and used as a target for drug discovery and for designing molecules that can prevent the virulence of EPEC. Preventing the virulence of EPEC would be an alternative to the traditional antibiotic-based approach to treating the illness, and in the long run, this could help to alleviate the problem of antimicrobial resistance.

Results and Discussion

The interaction between Tir and intimin is critical to EPEC pathogenicity, and the resulting pathological phenotypes may be analyzed using an imaging-based phenotypic assay. We hypothesized that small molecule compounds could prevent EPEC virulence by interfering with the normal binding of the ligand to the receptor. Using structural data from the crystal structure of the receptor and ligand complex (PDB entry 1F02), we first estimated the capacity of small molecules from large-scale virtual libraries to bind to three selected locations on these structures using docking studies. Following that, the most promising compounds were empirically tested using an actin pedestal counting assay with high-content imaging.

The Crystal Structure of Tir-Intimin Suggests Three Potential Sites for Binding of Inhibitors

The interface between intimin and its receptor, Tir, is a broad (1335 Å^2) and shallow protein-protein interface that is mainly hydrophobic (Figure 1).[5] The positively charged intimin tip, on domain 3, is formed by a short α-helix (residues 904–909). The helix is hydrogen-bonded to the main chain amides of Tir residues Asp 315 and Asp 316 via the main chain carbonyl oxygens of Ser 909 and Lys 908. Adjacent to these residues there is a β-sheet with similar characteristics and topology as the carbohydrate recognition site of C-type lectin domains. This area is known to participate in binding Tir.[9]

Monomers of the Tir intimin binding domain are hairpin-shaped molecules with two long α-helices connected via a β-hairpin. This hairpin structure is the main binding partner with intimin D3. Additionally, the two α-helices mediate dimerization by forming an antiparallel four-helix bundle similar to ColE1 Rop protein, a biomolecule responsible for maintaining *E. coli* plasmid copy number. This interface is also hydrophobic except for a pair of hydrogen-bonded Thr 282 side chain residues.

We selected three different sites in the complex as starting points for interfering with the interaction between the receptor and ligand. Firstly, 1) the intimin binding site on Tir, as this contains the hotspot for the PPI and previous studies suggest it to be required for function, second 2) the extracellular domain of intimin itself, as this is the opposing binding partner to the above mentioned binding site on the receptor and lastly 3) the receptor dimer interface (Figure 1B), as clustering of the receptors mediates their activity. Computational models for docking were developed for these sites as follows (Figure 1D–F).

Model 1. Intimin binding site on Tir

Tir acts as the receptor on the plasma membrane of the host cell. We ran virtual screening on a library of commercially available compounds to identify small molecules capable of disrupting the interaction between intimin and Tir. These compounds were docked into the binding site around the Asn 300 of Tir, which scanning alanine mutagenesis has shown to be a part of the binding hotspot, and thus important for interaction with intimin.[7] The model binding site was composed of residues Phe 291 and Asn 296 – Val 318. The Asn 300 side chain was set as a hydrogen bond donor and acceptor constraint in the docking calculations, therefore all hit compounds could form two hydrogen bonds with Asn 300 (Figure 1D).

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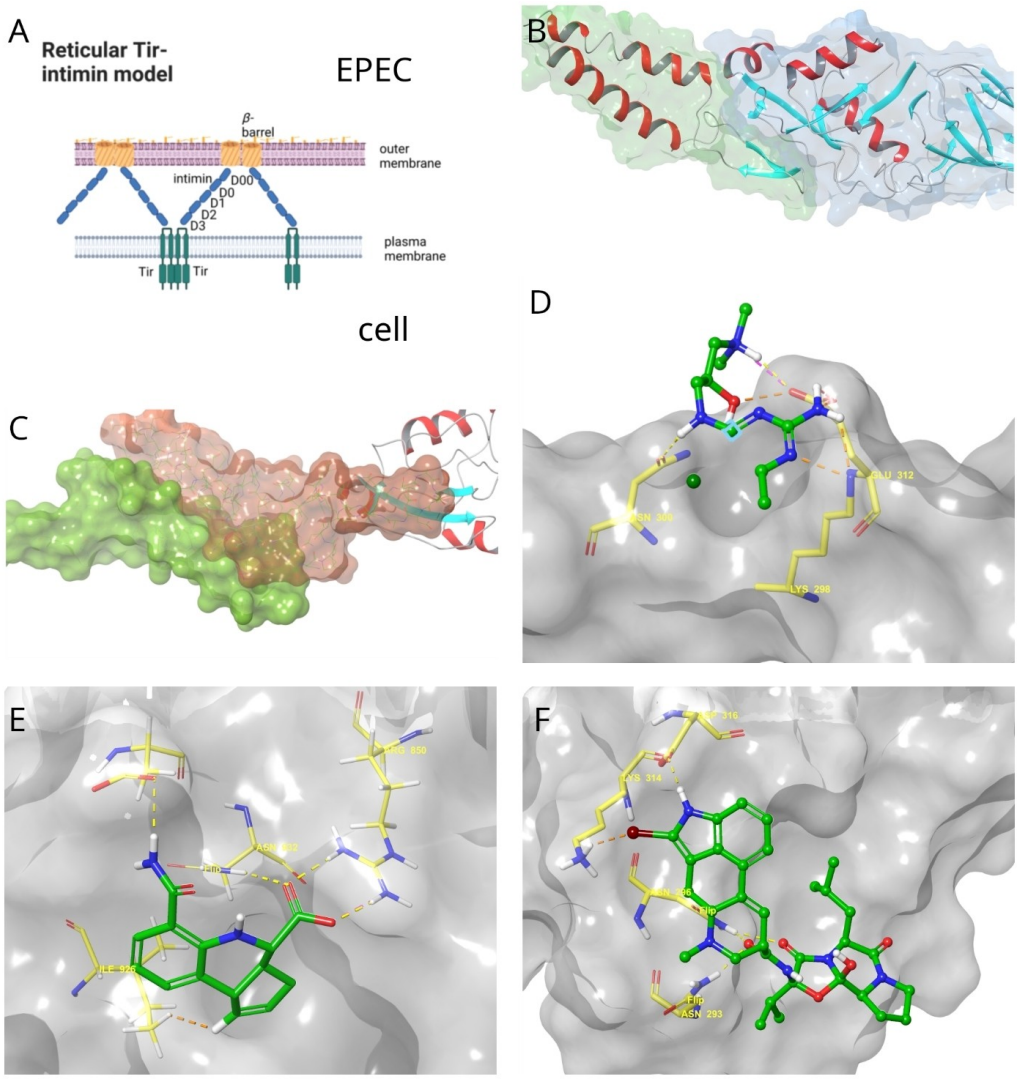


Figure 1. Intimin, a bacterial adhesin binds to a receptor that has translocated to the plasma membrane; A) A schematic representation of intimin and its receptor Tir. Both the adhesin and its receptor are believed to form dimers and bind to each other in a reticulating manner. Intimin consists of several domains (D00 to D3). The passenger domains of the adhesin are exported through the outer membrane and the extracellular lectin-like domain of intimin (D3, blue), interacts with the receptor (Tir, green), and the receptor clusters into a dimer. B) The interface between intimin (blue surface) and Tir (green surface), secondary structure as ribbon; C) The Tir-Tir interface, two instances of the Tir monomer in red and in green. The ribbon depicting an intimin unit; D) Compound 7 docked in Model 1 (Tir); E) Compound 39 in Model 2 (intimin D3 domain); F) Compound 48 in Tir-Tir interface (Model 3).

Model 2. Extracellular domain of intimin

As mentioned earlier, lectin-like intimin D3 domain acts as the natural ligand to Tir. We carried out virtual screening utilizing a library of compounds available from the Institute for Molecular Medicine Finland (FIMM) to identify small molecules capable of disrupting the interaction between intimin-Tir via binding to the extracellular domain of intimin. The compounds were docked to a site near the 890–930-residues, interacting with the short alpha helix or β-strands C, D, and E, relevant for binding Tir (Figure 1E) according to mutagenesis and yeast two-hybrid studies.[9]

Model 3. Receptor dimer interface

Because the crystal structure of Tir shows a dimer of the receptor molecules in an antiparallel orientation[5] and empirical

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research has shown that the receptors dimerize upon activation by intimin,[6] we hypothesized that a small molecule compound binding at the dimerization interface might inhibit the dimerization and thus prevent the pathological changes in actin dynamics. SiteMap was used to uncover a site on the receptor at the dimerization interface. To identify small molecules able to disrupt Tir-Tir clustering, we performed virtual screening using a library of compounds available from FIMM (Figure 1F).

Compounds from these models were selected for biological evaluation based on the scoring function, interactions formed at the binding site, binding site shape complementarity, and chemical structure (Table S1).

In Vitro Evaluation of the Selected Compounds

We employed an imaging assay based on the fluorescent actin staining assay adapted for high-content screening (FAS-HCS) to analyze the compounds identified via virtual screening.[10] In this assay, the actin binding molecule phalloidin is utilized to visualize EPEC infection *in vitro*, which is characterized by aberrant actin-rich structures on host cells known as pedestals. To visualize the bacterial colonies, an EPEC strain expressing mCherry is employed and, aurodox, a Tir translation inhibitor, is used as a control.

None of the compounds targeting the intimin binding site on Tir (Model 1) inhibited pedestals (Figure S2). One of the compounds (19) targeting the intimin lectin domain (Model 2) decreased the readout (Figure S3). However, closer examination revealed that the compound is fluorescent at the detection wavelength, interfering with the assay detection signal (Figure S4). Such interference at lower wavelengths is typical and should always be controlled for. Compounds targeting the receptor dimer interface (Model 3) were initially tested at two concentrations. At 50 μM, one compound (48) considerably and five (60, 66, 73, 74, and 75) marginally lowered the assay readout (Figure 2, Table S1), but did not do so at the lower concentration of 25 μM (Figure S5, Table S1).

Compounds 48, 60, 66, 74, and 75 were repurchased from commercial vendors for additional testing. Compound 73 was no longer available from the suppliers. Furthermore, compound 75 did not show a reproducible concentration-dependent impact, and compound 74 was a known cytotoxin (lanatoside C), so the two were excluded from further testing (Figure 3).

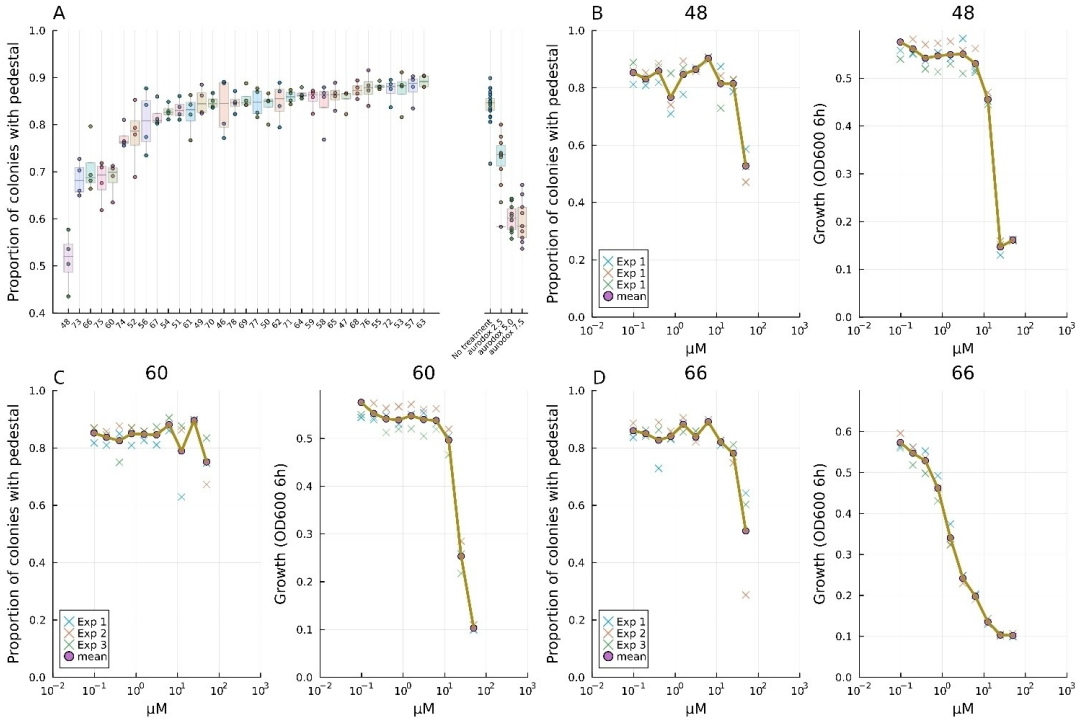


Figure 2. Six of the compounds (48, 73, 66, 75, 60, and 74) selected via the virtual screening model 3 targeting the receptor dimer interface decreased pedestals (50 μM). A) The FAS-HCS assay was run once with four technical replicates (n = 1). Aurodox was used as the positive control and 1% DMSO in MEM as the no treatment condition with 10 replicate wells each. Compounds are plotted in descending order based on activity. Three compounds with respective concentration response data from the FAS assay with growth inhibition at the same concentrations at 6 h; B) Compound 48; C) Compound 60; D) Compound 66. Measurements based on two technical replicates, the average of which indicated by an X for each three independent experiments (n = 3). The grand mean for these three is indicated by the purple circle. The concentrations range from 50 μM down in ten two-fold serial dilutions.

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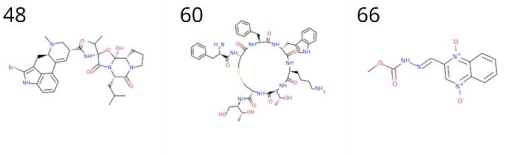


Figure 3. Structures of the three compounds (48, 60, and 66) selected from primary screening for evaluation in further experiments.

Known Antimicrobial Compounds at Subinhibitory Concentrations Do Not Prevent Pedestals

Some of the compounds selected by the virtual screening in model 3 were known antimicrobial compounds (79, 80, 81, 82, and 83) (Table S1). At these high concentrations antimicrobials abolish microcolonies required for image analysis. Therefore, we decided to test the capacity of these compounds to inhibit actin pedestals at multiple lower concentrations. Antimicrobials typically display polypharmacy, and previously described T3SS-inhibitors, such as aurodox, act as growth inhibiting antibiotics at higher concentrations.[11]

However, only the lowest concentration (95 nM) of compound 81 (ciprofloxacin) did not inhibit growth (Figure S7), although it did lower colony forming units significantly (Table S2). This lowest concentration showed no activity in inhibiting actin pedestals (Figure S7). Compound 80 (tomatine) also decreased pedestals significantly at concentrations only minorly affecting growth (Figure S7). Compounds 79, 82 and 83 fully inhibited growth even at the lowest tested concentration (95 nM), but did not show inhibition in the FAS-assay at this concentration.

Hit Compounds Inhibit the Growth of Bacteria or the Viability of Host Cells

Growth inhibition affects actin pedestal counts in the FAS-HCS assay, leading to false positive results. For example, antibiotic treated EPEC typically still adhere to cell monolayers to some extent, but their ability to establish microcolonies or produce actin pedestals is impaired. Microcolonies are also required to properly segment the image.

Therefore, we investigated whether the three hit compounds influenced bacterial growth with some non-specific toxic mechanism. At the primary screening concentrations (50 and 25 μM), all the hit compounds inhibited growth (Figure 2). These findings were further supported by colony plating experiments. The 50 μM treated plates had no colonies, but the 25 μM treated plates had low colony counts, indicating toxicity (Tables S2A and S2B).

Pedestal inhibition, just as the prevention of any other cellular phenotype, may also result from a decrease in host cell viability, especially at high concentrations of compounds more likely to display cytotoxicity. As a result, tests were conducted to investigate the cytotoxicity of compounds that decreased the readout in the primary screening at high concentrations (50 μM). Except for compound 80 (tomatine) at the maximum 50 μM concentration, none of the compounds reduced the viability of the host cells (Figure S6).

Conclusions

In this study we searched for inhibitors of the translocated intimin receptor of EPEC. We utilized computational chemistry methods to select compounds potentially capable of interfering with the receptor complex and then tested these using a robust screening assay based on widely used FAS-assay and automated image analysis. Selective inhibition of pedestals via inhibiting Tir could prove to be a medically efficient strategy to treat EPEC infections, but there are currently no known inhibitors of this receptor.

Our work identified some challenges in interfering with this pathway. The crystal structure of the protein complexes does not appear to have a deep pocket or clefts found on many drug targets such as on G-protein coupled receptors, kinases, other enzymes. We demonstrated that the used methodology is not sufficient to uncover small molecule inhibitors of the translocated intimin receptor. Without any good structure-based information to go on, the best way forward would be to use a very comprehensive search, e.g., by screening large biogenic compound collections.

Small molecule inhibitors of Tir would be a new class of therapy for infections of A/E pathogens. Currently there are no A/E pathogen infection specific therapies.

Overall, antivirulence therapies could avoid the issues in traditional antibiotic treatments, both the following adverse effects and the increase of antimicrobial resistance, thus giving new opportunities to tackle the problem of antimicrobial resistance.

Experimental Section

Virtual Screening and Selection of Compounds

A 2.9 Å resolution crystal structure of the C-terminal 282-residue fragment of intimin in complex with the intimin-binding domain of translocated intimin receptor (PDB code 1F02) was used for all the models.

Model 1. Intimin binding site on Tir

Compound library

Drug-like small molecule libraries from Asinex, ChemBridge, Enamine, Life chemicals, Key Organics, and Vitas-M were downloaded in SDF format. These libraries were merged, duplicates were removed, and the correct protonation and tautomeric states were assigned which resulted in a library containing 741 778 compounds. For these compounds a library of conformers was generated using the OMEGA software (Release 4.1.1.1, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com)[11]

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using default settings, which resulted in a maximum of 200 conformers per ligand.

A collection of compounds available at the Institute for Molecular Medicine Finland (FIMM), University of Helsinki, in a database of 132 566 compounds was prepared using LigPrep by including options such as adding hydrogens, generating ionization states at pH 7.2 ± 2.0, generation of tautomers, and multiple conformers. This library has been generated from multiple sources, from commercial libraries (Microsource, SPECTRUM, NIH clinical collection, diversity sets from ChemDiv and ChemBridge, peptidomimetic compounds, and FDA-approved drugs). The protein preparation workflow of Maestro Suite was used to prepare the intimin protein domain, by separating it from the cocrystal structure (1F02), correcting errors in the structure, such as adding missing hydrogens and minimization of hydrogen atoms, filling missing side chains.

Structure-based virtual screening

For preparing the binding site, the above-mentioned crystal structure was used, and the binding site was prepared using MAKE RECEPTOR (Release 4.1.0.1, OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com). The grid box around the Asn 300 residue of Tir was generated and adjusted to get a box with the following dimensions: 17.33 Å×26.33 Å×14.67 Å and the volume of 6694 Å^3. For "Cavity detection" the slow and effective "Molecular" method was used for detection of binding sites. Inner and outer contours of the grid box were also calculated automatically using "Balanced" settings for "Site Shape Potential" calculation. The inner contours were disabled. Asn 300 was defined as a hydrogen bond donor and acceptor constraint for the docking calculations. The small molecule library, prepared by OMEGA, was then docked to the prepared binding site on intimin (PDB entry: 1F02)[5] using FRED (Release 4.1.0.1. OpenEye Scientific Software, Inc., Santa Fe, NM, USA; www.eyesopen.com)[12,13] Docking resolution was set to high, other settings were set as default. A hit list of the top 1 000 ranked molecules was retrieved and the best ranked FRED-calculated pose for each compound was inspected visually and used for analysis and representation.

Model 2. Extracellular domain of intimin

Compound library and protein preparation

The same collection of compounds available at FIMM was used similarly as above (Model 1). The protein preparation workflow of Maestro Suite (version 2018-1, Schrödinger) was used to prepare the Tir-binding domain of intimin, by separating it from the cocrystal structure, correcting errors in the structure, such as adding missing hydrogens, and minimization.

Structure-based virtual screening

For the binding site preparation, the above-mentioned crystal structure was used, and a grid box was created by Glide[14] grid generation around the alpha helix (residues 904–909) of intimin with dimensions 20 Å×20 Å×20 Å.

Docking studies were carried out to identify possible interacting ligands using the Glide virtual screening workflow implemented in Schrödinger. Virtual screening workflow of Glide includes various stages with increasing docking precisions such as high-throughput virtual screening (HTVS), standard prediction (SP), and extra precision (XP) mode. All molecules were processed initially in HTVS mode and the top 10% of HTVS output was processed with SP docking mode. Then, the top 10% molecules of SP docking were processed through XP docking.

Model 3. Receptor dimer interface

Compound library and protein preparation

The same collection of compounds available at FIMM was used similarly as above (Model 1). The protein preparation workflow of Maestro Suite was used to prepare the extracellular domain of Tir, by separating it from the cocrystal structure, correcting errors in the structure, such as adding missing hydrogens, and minimization.

Structure-based virtual screening

Schrödinger Sitemap[15] was used to discover a site with Dscore 0.47 using the same PDB entry as in Model 2. Using the default settings for SiteMap, only this one site was discovered on the receptor. Using the shallow setting, a site with a Dscore of 0.97 was in the same cavity, so we decided to use this. However, the Glide grid was based on the site located using the default settings. This site is located next to the Trp 285 residues that form a hydrogen bond in the dimer interface.

Using the Glide virtual screening workflow built in Maestro, docking tests were performed to identify potential interacting ligands. All molecules were originally processed in HTVS mode, with the top 10% of HTVS output being processed in SP docking mode. The top 10% of SP docking molecules were then processed through XP docking.

High-content Imaging and Screening

Primary screening and concentration-response experiments were performed using a modification of the widely used FAS assay published earlier.[10] In short, this is an infection assay with fluorescence imaging readout. A cell monolayer is infected with an EPEC strain emitting fluorescence and host cell actin is stained with phalloidin. The data is processed using a custom data reduction pipeline which produces as a readout the proportion of all bacterial microcolonies with actin pedestals. This is achieved by segmentation algorithms and a colocalization analysis. The code used for analysis is available at https://github.com/tpylkko/FAS-HCS.

Compound Sources

For testing compounds from docking Model 1 the compounds were purchased from Molport, and 10 mM stocks in DMSO were prepared and stored at −20 °C until use. The stocks were serially diluted to source plates (384-well, Nunc) using Biomek i7 liquid handling workstation (Beckman Coulter) in a volume of 37.5 μL.

For testing compounds from Models 2 and 3, compounds were plated to 384-well plates by using an Echo liquid handler (Labcyte) at the Institute for Molecular Medicine Finland (FIMM), High Throughput Biomedicine unit. Before use, 37.5 μL of MEM was dispensed to these plates and they were shaken at RT for 30 min (500 rpm).

For retesting compounds for concentration-response, compounds were reacquired from Molport and stocks of 2.5 mM or 10 mM were prepared in DMSO. The stocks were serially diluted to source plates (384-well, Nunc) using Biomek i7 in a volume of 37.5 μL. Two dimensional representations of all these compounds are available in Scheme S1.

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Screening Procedure

To screen the compounds, 37.5 μL of preincubated EPEC (2348/69) suspension was added to the above-mentioned source plates, containing the compounds in 37.5 μL of MEM with a dispenser (Mantis, Formulatrix) in such a way that in the end the source plate contained 75 μL of a mixture with the correct concentration of the compounds and a bacterial suspension with a multiplicity of infection of 1 : 15. A volume of 60 μL of this mixture was moved to the screening plates containing Caco-2 (HTB-37 ATCC) cells and the plates centrifuged at 1000 g for four minutes and moved to 37 °C, 5 % CO_2 for two hours (Biospa, Biotek). After this, an equal volume (60 μL) of the 2X staining solution was applied to the plate using a dispenser (Mantis, Formulatrix) and incubated at RT for 20 minutes. Biomek i7 was used to wash the plate three times with HBSS and the imaging was then done on the Cytation 5 (Biotek). More details are provided in the previously published protocol.[10]

Bacterial and Cellular Viability Assays

EPEC 2348/69 was grown overnight in LB liquid culture. Compounds were plated onto a 384-well plate in LB or Miller Hinton broth and shaken (500 rpm, 30 min) using a Biomek i7 protocol. The bacterial suspension was then diluted to the appropriate range (2 million CFU ml^{-1}) by measuring the optical density using a densitometer. This suspension was dispensed using a dispenser (Mantis, Formulatrix) into the compound plate to reach a total volume of 75 μL. After this, the plates were incubated using Biospa (Biotek) for 24 hours and their OD_{600} measured once an hour using Cytation 5. This procedure was repeated three times using a different colony and newly prepared media and other reagents, and the well location of a specific compound series was varied among the repeats randomly to diminish any possible plate area effects.

To measure CFU counts, a similar procedure was used, except that after treatment of two hours, a 3 μL sample of bacteria was taken from each well and diluted by a factor of 30 into a new 96-well plate containing PBS. This step was repeated to reach a total dilution factor of 900. From this suspension, 50 μL samples were spread on LB agar plates and incubated at 37 °C overnight. Images were captured of the plate using a digital camera from a phone. The number of colonies on plate was then calculated using OpenCFU 3.9. Three independent experiments were performed on distinct days using distinct colonies, and newly prepared media and reagents.

Cellular viability was assessed using the CellTiter-Glo (Promega) kit according to the manufacturer's instructions. Assay conditions were identical to the screening procedure described above except that 0.5 % Triton-X was used as the positive control and PBS as the negative. Three independent experiments were performed on distinct days using newly acquired media and reagents.

Author Contributions

Tuomas Pylkkö: Conceptualization, Methodology, Software, Data curation, Writing – Original draft preparation, Visualization, Investigation, Validation, Writing – Review & Editing; Tihomir Tomašič: Investigation, Methodology, Writing – Review & Editing; Antti Poso: Supervision, Writing – Review & Editing; Päivi Tammela: Conceptualization, Supervision, Writing – Reviewing and Editing, Funding acquisition.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: EPEC · high-content imaging · intimin · translocated intimin receptor

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Bioprospecting of inhibitors of EPEC virulence from metabolites of marine actinobacteria from the Arctic Sea

Tuomas Pylkkö[1], Yannik Karl-Heinz Schneider[2], Teppo Rämä[2], Jeanette Hammer Andersen[2] and Päivi Tammela[1]*

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A considerable number of antibacterial agents are derived from bacterial metabolites. Similarly, numerous known compounds that impede bacterial virulence stem from bacterial metabolites. Enteropathogenic *Escherichia coli* (EPEC) is a notable human pathogen causing intestinal infections, particularly affecting infant mortality in developing regions. These infections are characterized by microvilli effacement and intestinal epithelial lesions linked with aberrant actin polymerization. This study aimed to identify potential antivirulence compounds for EPEC infections among bacterial metabolites harvested from marine actinobacteria (*Kocuria* sp. and *Rhodococcus* spp.) from the Arctic Sea by the application of virulence-based screening assays. Moreover, we demonstrate the suitability of these antivirulence assays to screen actinobacteria extract fractions for the bioassay-guided identification of metabolites. We discovered a compound in the fifth fraction of a *Kocuria* strain that interferes with EPEC-induced actin polymerization without affecting growth. Furthermore, a growth-inhibiting compound was identified in the fifth fraction of a *Rhodococcus* strain. Our findings include the bioassay-guided identification, HPLC-MS-based dereplication, and isolation of a large phospholipid and a likely antimicrobial peptide, demonstrating the usefulness of this approach in screening for compounds capable of inhibiting EPEC virulence.

KEYWORDS

antivirulence, EPEC, arctic marine microorganisms, bioprospecting, actinobacteria

1 Introduction

The antimicrobial crisis is the result of the convergence of two phenomena. To begin with, there have been few pharmaceutical antibiotic breakthroughs in recent decades (World Health Organization, 2022). Second, there are reports of increasingly troublesome cases of antibiotic resistance, globally already contributing to millions of deaths annually (Murray et al., 2022). Historically, researchers have sought antibacterial compounds in natural products, particularly in other microbes (Schneider, 2021). And this has had a high success rate; in fact, soil actinobacteria have produced 80% of all currently licensed antibiotics. However, marine actinobacteria found in the sea, on the seafloor or within the microbiome of marine organisms have received far less attention as possible sources of antibiotics, even more so with respect to virulence-modifying compounds.

Inhibiting bacterial virulence is a well-studied alternative method to the more traditional killing of microorganisms or inhibiting their growth (Zambelloni et al., 2015; Defoirdt, 2016; Buroni and Chiarelli, 2020). In essence, the idea is to inhibit the action of virulence causing molecules using pharmaceutical interventions. In the best-case scenario, the treated pathogens would then remain incapable of causing symptoms, but nevertheless alive, and thus selection pressure for resistance would not form so easily. Furthermore, due to their specificity, such drugs would most likely have fewer adverse effects on normal flora, which are affected adversely by drugs inhibiting bacterial growth or viability in general. Many compounds that to date have been described to be able to prevent bacterial virulence have been discovered from natural sources using phenotypic screening assays (Kimura et al., 2011; Duncan et al., 2014; Wu et al., 2019; Mühlen et al., 2021). These include inhibitors of the expression of virulence molecules, inhibitors of the translocation of effectors, pilicides, and adhesion blockers.

Enteropathogenic *Escherichia coli* (EPEC) is a gram-negative bacterium responsible for a significant portion of diarrheal illnesses and mortality in children under five worldwide (Ochoa and Contreras, 2011). EPEC isolates also display many different forms of antimicrobial resistance, including fluoroquinolone-resistance (Eltai et al., 2020), plasmid-mediated carbapenem and colistin resistance, and extended spectrum betalactamases (Karami et al., 2017; Mahmud et al., 2020). EPEC virulence is caused by it adhering to enterocytes and causing lesions in the intestinal epithelium characterized by the destruction of microvilli, a phenomen called attaching and effacing (A/E) lesions (Kaper et al., 2004). Once adhered to the cell, EPEC employs a type III secretion system (T3SS) to deliver various virulence factors into host cells that use the cell's own actin nucleation machinery to induce pathological changes in the cell (Campellone et al., 2002). Among the secreted factors is the translocated intimin receptor (Tir), which is critical for A/E lesion formation (Cleary, 2004). The receptor's ligand is a protein autotransported by the bacterium to its outer membrane, facilitating intimate attachment to the host cell. Once in place, the phosphorylation of the receptor initiates the recruitment of NCK, N-WASP and the Arp2/3 complex leading to abnormal actin polymerization and actin-rich protrusions on the plasma membrane (pedestals) beneath adherent EPEC (Deborah Chen and Frankel, 2005). Consequently, targeting this process holds promise for the development of antivirulence therapies, and it could be inhibited at various different stages of the pathway, for example, by preventing the contact of Tir and its ligand, intimin by orthostatic inhibition of the receptor, via down regulation of the virulence factors, or inhibition of transport via the T3SS.

As a first step toward discovering antivirulence compounds for EPEC infections, we studied the effects of extracts and fractions from four marine actinobacteria for the ability to decrease Tir-mediated virulence and the following abnormal actin condensation within the cells. Additionally, we sought to evaluate the suitability of these assays for screening bacterial extract fractions with a complex mixture of compounds, including potential pan-assay interference compounds (PAINS). The actinobacteria were isolated from sampling sites near Svalbard in the Arctic Sea and identified as using 16S marker gene sequencing. Next, they were cultured in artificial media, extracted for secondary metabolites, and the extracts fractionated for studying their effects against EPEC caused virulence *in vitro*. Three bioactivity screening methodologies were used for each extract. These included (1) testing for their capacity to inhibit the translocation of Tir, (2) their capacity to prevent actin pedestals, and (3) their capacity to inhibit the growth of EPEC in liquid culture. The recognized active fractions were then studied further to narrow down their possible mechanism of action and to elucidate the chemical structure of the active compounds.

Our aim was to design and validate an isolation and automated screening workflow for use with fractions from microbial cultures and explore the presence of virulence-inhibitory compounds within marine bacterial fractions and their potential application for drug development as the complex nature of extracts and extract fractions may interfere with screens that have been developed and validated using pure chemical compounds only. First due to the complex mixtures potentially containing a high number of "promiscuous binders" or pan assay interfering compounds (PAINS), but also due to the high concentration (10–100 μg extract/fraction per mL) commonly tested in initial screens. This requires (1) assaying methodology suitable for high-throughput screening (2) that can be used with complex fractions, not only pure compounds and (3) methodology to isolate and recognize which constituents are the active ones. We show that this workflow can indeed recognize bioactive compounds in these microbial fractions. In addition, the specific inhibition of enteropathogenic *Escherichia coli* (EPEC) virulence could offer an alternative to conventional antibiotic-based approaches, helping to mitigate the issue of antimicrobial resistance over the long term.

2 Materials and methods

2.1 Chemicals and reagents

pH_2O was produced by the in house MilliQ system (Merk, Millipore), methanol (HiperSolv, VWR) and acetone (HiperSolv, VWR), dimethyl sulfoxide (DMSO, VWR) were used if not otherwise indicated.

Modified marine ISP2 medium was produced using 4.0 g glucose (Sigma Aldrich) 4.0 g yeast extract (Sigma), 10.0 g malt extract (Sigma), 300 mL filtered seawater, 700 mL dH_2O, and a trace element solution 0.2% (v/v). Filtered seawater was produced by the seawater supply of the Norwegian college of fishery science in Tromsø, Norway, by filtration through a 0.22 μm Millidisk® 40 filter-cartridge (Millipore). The trace element solution was prepared by dissolving 10% $MgSO_4$ 7 × H_2O, 0.01% $FeSO_4$ 7 × H_2O, 0.01% $ZnSO_4$ 7 × H_2O, 0.01% $CuSO_4$ 5 × H_2O, and 0.01% CoCl2 6 × H_2O, in pH_2O, (w/v). The media was autoclaved at 121°C for 30 min using an autoclave (MLS-3781L, Panasonic).

2.2 Bacterial strains, culture, and extraction

For the infection model, the EPEC E2348/69 from Bacteriology reference department (BRD) (UK), was used. The strain was transformed using the plasmid pON.mCherry, Addgene #84821

deposited by Howard Schuman, constitutively expressing mCherry, a fluorescent protein, and grown on LB agar plates or LB broth supplemented with 30 μg mL^{-1} chloramphenicol at 37°C and 200 rpm. For more details (see Pylkkö et al., 2021).

For Tir translocation assays, an EPEC E2348/69 strain containing a beta-lactamase chromosomal fusion in LEE5 (for Tir) under the control of the native promotor, was used (CX2135) (Mills et al., 2008). This strain was kindly provided to us by Ilan Rosenshine from the Hebrew University of Jerusalem. This was cultured on LB agar plates or LB broth supplemented with 50 μg mL^{-1} tetracycline at 37°C and 200 rpm.

The actinobacteria strains were isolated from animals collected in the Arctic Sea in August 2020 (Schneider et al., 2022) (listed in Table 1). For the screening of bioactivity, 2 × 500 mL of the strains were cultivated and extracted as described below. For the isolation of compounds, the strains T091 and T160-02 were cultured in 6 × 500 mL modified marine ISP2 medium for 14 days at 20°C and 140 rpm using a shaking-incubator (Multitron Pro, INFORS HT). For extraction, 40 g of Diaion® HP20 Resin (Merck) was used. The resin was activated by incubation in methanol for 30 min and washed with pH_2O, for 20 min before the resin was added to the cultures and incubated for 3 days. The resin was separated from the cultures using vacuum filtration and cheese cloth filter (1057, Dansk Hjemmeproduktion). The pooled resin for each strain was extracted two times using 2 × 300 mL of methanol for 45 min of extraction. The extract was separated from the resin using Whatman No.3 filter paper and vacuum filtration. The extract was dried *in vacuo* at 40°C.

The exact contents of the used growth media are listed in Supplementary Table S1.

2.3 Preparation of fractions

Crude extracts were fractionated using flash liquid chromatography. The extracts were loaded onto resin (Diaion® HP-20ss, Supelco) by dissolving them in 90% methanol aq. (v/v) and adding resin in a ratio of 1:1.5 (resin/dry extract, w/w). Subsequently, the solution was dried under reduced pressure at 40°C. Flash columns (Biotage® SNAP Ultra, Biotage) were prepared by activating the resin by incubation in methanol for 20 min, washing with ddH_2O, and loading it into the column ensuring the resin being always covered with water. 6.0 g HP-20ss resin was loaded on one column. The fractionation was performed using a Biotage SP4™ system and a water: methanol gradient from 5–100% methanol over 36 min (6 min 5% B, 6 min 25% B, 6 min 50% B, 6 min 75% B, 12 min 100% B) followed by a methanol: acetone step-gradient (4 min methanol, 12 min acetone). The flow rate was set to 12 mL/min. Twenty-seven eluent fractions of 24 mL each were collected in glass tubes and pooled into six flash fractions in total (1–3 were pooled to fraction 1; 4–6 to fraction 2; 7–9 to fraction 3; 10–12 to fraction 4; 13–15 to fraction 5; 16–27 to fraction 6). An appropriate amount of extract-resin mixture was loaded onto the column after equilibration to 5% methanol aq. (v/v). The flash fractions were dried under reduced pressure at 40°C.

2.4 Analysis of fractions using HPLC-HR-MS2

For HPLC-HR-MS2 analysis an Acquity I-class UPLC (Waters) was used coupled to a PDA detector and a Vion IMS QToF (Waters). The HPLC was equipped with a Acquity C-18 UPLC column (1.7 μm, 2.1 × 100 mm) (Waters). The mobile phases consisted of acetonitrile (HiPerSolv, VWR) for mobile phase A and pH_2O as mobile phase B, both containing 0.1% formic acid (v/v) (33015, Sigma). The gradient was run from 10 to 100% B over 13 min at a flow rate of 0.45 mL/min. Samples were run in ESI+ and ESI- ionization mode. The data was processed and analyzed using UNIFI 1.9.4 (Waters). Exact masses were calculated using ChemCalc (Patiny and Borel, 2013). For dereplication an extract of modified marine ISP2-medium and flash fractions of the extract were prepared and analyzed using the same HPLC-MS2 method in order to exclude media components from consideration. (i) PubChem (Kim et al., 2023) and (ii) Chemspider (Pence and Williams, 2010) where used to identify potential compounds during dereplication by elemental composition search (i + ii) and MS-fragment search (ii, implemented in UNIFI).

2.5 Isolation of compounds using RP-HPLC-MS via mass triggered fractionation

For the isolation of compounds from flash fractions preparative reversed phase HPLC was used. Fractionation was triggered by the recorded mass signal throughout the chromatographic separation. The HPLC system consisted of a Waters 600 HPLC-pump with a degasser and flow-splitter, a Waters 515 HPLC-pump as a "make-up" pump, a Waters 3100 Mass detector, a Waters 2996 photo array detector and a Waters 2767 sample manager (all Waters). The system was controlled using MassLynx V4.1 (Waters) software A Sunfire RP-18 preparative column (10 μm, 10 × 250 mm) and XSelect CSH preparative Fluoro-Phenyl column (5 μm, 10 × 250mm) (both Waters) were used as solid phases for the first and second round of purification, respectively. The mobile phases for the gradients were A [pH_2O with 0.1% (v/v) formic acid] and B [acetonitrile with 0.1% (v/v) formic acid]. The flow rate was set to 6 mL/min. Acetonitrile (Prepsolv®, Merck) and formic acid (33015, Sigma) were purchased in appropriate quality, ddH_2O was produced with the in-house Milli-Q® system. For the MS-detection of the eluting compounds one percent of the flow was split and blended with 80% MeOH in pH_2O (v/v) acidified with 0.2% formic acid (Sigma) and directed to the ESI-quadrupole-MS. The fractions were collected by mass triggered fraction collection and the respective fractions were reduced to dryness under reduced pressure and by vacuum centrifugation, both at 40°C.

2.6 Fluorescent actin stain assay

Screening was performed using a modification of the widely used FAS assay published earlier at various concentrations (Pylkkö et al., 2021). In short, this is an imaging-based infection assay. A

TABLE 1 Actinobacteria isolates investigated.

Strain ID:	T091	T289	T060	T160-2
Animal/origin	*Caulophacus arcticus* (*Porifera*)	*Chlamys islandica* (*Mollusca*)	*Dendrobeania sp.* (*Bryozoa*)	*Tricellaria ternata* (*Bryozoa*)
Actinobacterium	*Kocuria* sp.	*Rhodococcus* sp.	*Rhodococcus* sp.	*Rhodococcus* sp.
Location/depth	77.37491402 °N, 8.268010617 °E/1,400 m	74.78804448 °N, 8.57096443 °E/285 m	75.16057145 °N, 13.71765962 °E/1,500 m	75.16057145 °N, 13.71765962 °E/1,500 m
GenBank accession no.	OP537112.1	OP537141.1	OP537103.1	OP537122.1

More detail on the field sites and methodology used to isolate and culture the strains can be found in Schneider et al. (2022).

cell monolayer of 2×10^5 Caco-2 cells mL^{-1} (ATCC CCL-23) is infected with EPEC E2348/69 emitting fluorescence (mCherry) at a MOI of 1:15. This MOI has been determined to be appropriate for clearly distinquizable EPEC-mCherry microcolonies to form during the infection. Following this, actin is stained with phalloidin and nuclei with Hoechst 33342. For each well five fields of view are collected, and all well-level data are mean aggregates of the data from five fields of view. The data is processed using a custom data reduction pipeline which produces as a readout the proportion of all bacterial microcolonies with actin pedestals. This is achieved by segmentation algorithms and a colocalization analysis, each image is segmented into the microcolonies and features are extracted from the channels of both the bacteria and cells within these segments. The main readout produced by this is the proportion of segmented microcolonies that is associated with actin condensation, although other readouts are collected, such as the number and size of microcolonies. The images were analyzed using the custom scripts on a high-performance computing cluster, Puhti, provided by CSC—IT Center for Science, Finland. The code used for analysis is available at https://github.com/tpylkko/FAS-HCS.

To screen the samples, minimum essential media (MEM) preincubated (1:50, 3 h) mCherry-EPEC suspension at 2 × concentration was added to 96-well source plates (NUNC) using a dispenser (Mantis, Formulatrix), and the fractions dissolved in 2.5% DMSO-MQ were added in 2 × concentrations so that the correct concentration of the samples and bacterial suspension with a multiplicity of infection of 1:15 was achieved in the source plate. A volume of 60 μL of this mixture was transferred to the screening plates (Phenoplate 384, PerkinElmer) and the plates were centrifuged at 1,000 × g for 4 min to allow the bacteria to come into contact with the cells. The plates were subsequently incubated for 2 h at 37°C, 95%, humidity 5% CO_2 (Biospa, Biotek). After this, a staining solution was applied to the plate using a dispenser (Mantis, Formulatrix) and incubated at RT for 20 min. The plate was then washed three times (100 μL) with Hanks buffered saline solution (HBSS) with an automated liquid handling workstation (Biomek i7, Beckman Coulter) and imaged using a protocol in the imaging plate reader Cytation 5 (Biotek). The contents of this solution are in the Supplementary Table S2. More details about image capture techniques are in Pylkkö et al. (2021).

2.7 Tir translocation assay

For translocation assays, 2×10^5 Caco-2 cells mL^{-1} (ATCC CCL-23) were seeded into black 384-well plates with transparent bottom (Phenoplate, PerkinElmer, Germany). Bacterial overnight cultures, grown in 50 μg mL^{-1} tetracycline, were diluted 1:50 into MEM with GlutaMAX (Gibco, Germany) and incubated for 2 h at 37°C 5% CO_2 in a filter capped 50 mL Falcon tube. Hundred microliter of the bacterial suspension was subsequently added to a source plate (96-well, NUNC) to which samples were serially diluted. These plates were incubated for an additional 1 h. Caco-2 cells were washed once with HBSS. The bacteria-sample suspension (60 μL per well) was then added to the cells, the plates were centrifuged at 1,000 × g for 4 min (Eppendorf Centrifuge 5810R) and incubated for 1.5 h (37°C, 5% CO_2). Media was then removed, and infected cells were washed twice with 60 μL HBSS. MEM with 100 μgmL^{-1} gentamicin was added to the cells and mixed with LifeBLAzer CCF4-AM staining solution (Invitrogen). The plates were then incubated for 1 h at room temperature. Subsequently, the fluorescence was determined in a Cytation 5 (Biotek, Germany) using an excitation wavelength of 405 nm (10 nm bandwidth). Emission was detected with 460 and 530 nm. Effector translocation was determined by calculating the ratio of blue to green fluorescence (Em520 nm/Em460 nm) following the manufacturer's instructions.

2.8 Red blood cell hemolysis assay

EPEC overnight cultures were diluted 1:25 in DMEM high glucose without phenol red (Gibco) and grown for 3 h at 37°C, 5% CO_2 in the presence or absence of decreasing concentrations of the fractions. Red blood cells (RBC) were purified from Defibrinated Oxoid Sheep Blood (Thermo Fisher Scientific) by three rounds of centrifugation in 1.5 mL eppendorf tubes using a table top centrifuge at 2,000 × g and washing with PBS, then resuspended to 5% (v/v) in DMEM high glucose without phenol red. Bacterial cultures were equalized to 10^8 in 100 μL and added to 100 μL sheep RBCs (5% v/v) in a 96-well plate. Uninfected RBCs in DMEM were used as a negative control. Total lysis was achieved by adding 0.5% Triton-X to the culture medium. To synchronize infection and mediate bacterial-cell contact, tubes were centrifuged 1 min at 3,220 × g before incubation at 37°C, 5% CO_2. After 2 h, cells were gently resuspended, followed by centrifugation at 3,220 × g for 1 min. Fifty microliter of each supernatant was transferred to a 96-well plate, and the amount of hemoglobin released was assessed at 543 nm in Cytation 5 (Biotek) plate reader. Hemolysis was calculated as the percentage of hemoglobin released by the DMSO-treated wild-type-infected RBCs.

2.9 Analysis of the inhibition of growth of EPEC

Wild-type EPEC E2348/69 was grown overnight in LB broth at 37°C, 200 rpm and resuspended to 2×10^6 cells mL^{-1}. For the assay, 100 μL were added to each well of a 384-well plate containing appropriate amounts of sample or gentamicin 4 μg mL^{-1} as a control. Plates were incubated at 37°C, 95% humidity without shaking (Biospa, Biotek) and the OD_{600} was determined every hour for 24 h using Cytation 5 (Biotek).

3 Results

The actinobacteria strains investigated in this study were obtained during a research expedition aboard the Norwegian research vessel Kronprins Haakon in the Arctic Sea. These strains were isolated from samples of invertebrates (Schneider et al., 2022) (see Table 1, Section 2.2) Bacterial strains, cultures and extraction). After collection, the isolates were cultured in marine modified ISP2 media. Subsequently, they were subjected to solid-phase extraction using HP20-resin, followed by fractionation into six fractions via FLASH liquid reversed-phase chromatography. These fractions were then screened using various EPEC virulence related *in vitro* assays and further investigation was conducted on the bioactive fractions to identify the active compounds responsible for these effects (see Figure 1 for overview of workflow).

3.1 Fractions 5 from a *Kocuria* sp. and a *Rhodococcus* sp. reduce actin pedestals

To evaluate if the bacterial extract fractions suppress the establishment of actin pedestals, a hallmark of EPEC infections, we employed a high-content screening format of the commonly used fluorescent actin staining (FAS-HCS) assay (Knutton et al., 1989; Pylkkö et al., 2021). When EPEC infects host cells it causes actin-rich pedestals in the vicinity of the microbial microcolonies adhered to the host cells (Campellone et al., 2002). These structures—often called pedestals in the literature—can be visualized using actin staining in cell culture monolayers by phalloidin conjugated fluorophores. This assay additionally quantifies the counts and size of EPEC microcolonies. Previous research in our labs has indicated that these readouts can be used to recognize known antibiotics, as microcolony counts and size tend to show dramatic decrease with these treatments (Pylkkö et al., 2023). Bacteria are typically not entirely killed (and washed away) by short-term treatment with antibiotics, particularly at lower concentrations.

Fractions originating from two of the bacteria (fractions 5 of T091 *Kocuria* sp. and T160-2/*Rhodococcus* sp.) reduced actin pedestals (Figure 2A). The subsequent fraction (fr.6) also had a similar, but weaker effect, likely due to the fact that they contain the same compounds in lower quantities. The other fractions did not display such activity (Supplementary Figure S1). A reduction in the assay readout can occur due to mechanisms relating to bacterial virulence, such as the constituents inhibiting the adhesion of EPEC to the cells, or down regulating virulence related proteins. However, it can also occur due to other indirect mechanisms such as the compounds being toxic to the organisms, or somehow obscuring the image processing pipeline. Therefore, further studies were conducted.

3.2 Fractions 5 from a *Kocuria* sp. and a *Rhodococcus* sp. also reduce Tir translocation

In order to cause pathological changes, such as A/E lesions and associated actin pedestals, EPEC injects virulence factors (e.g., Tir) into the host using a molecular syringe-like device called the type three secretion system. The efficiency of virulence molecule translocation has been studied using beta-lactamase reporter fusions. In this high-throughput method, one C-terminally tags the effector of interest (Tir in this study) with the TEM-1 β-lactamase and infects cells loaded with a FRET signal capable molecule (CCF2-AM) (Mills et al., 2008). CCF2-AM is a molecule in which the donor and acceptor fluorophores are linked together with a beta-lactam. Therefore, the signal emitted by the cells correlates with the degree to which the reporter fusion enzyme (beta beta-lactamase) has cleaved the intracellular molecule, and thus, indirectly with the efficiency of translocation. Beta-lactamase enzymes will not normally be present in unmodified cultured human cell lines. The marine bacterial fractions previously recognized as inhibiting actin pedestals (T091-5 and T160-2-5) also reduced the translocation efficiency of Tir to the infected cells in a concentration dependent manner in this assay (Figure 2B).

3.3 Fractions from a *Rhodococcus* sp. T160-2 prevent red blood cell hemolysis

EPEC infection causes rapid red blood cell hemolysis, and this is likely caused by the injection of EspB and EspD proteins that form a pore on the plasma membrane, which is then used by the type three secretion needle complex to inject molecules into the host cell (Luo and Donnenberg, 2006). Some compounds, such as aurodox for example, can prevent hemolysis efficiently even if they may only modestly prevent actin pedestals (Kimura et al., 2011; Pylkkö et al., 2021). Nevertheless, in *in vivo* murine infection experiments with *Citrobacter rodentium*—a murine specific A/E-pathogen—aurodox protected the entire treatment cohort from death, while all individuals were lost in the no treatment condition and antibiotic treated condition by day 13 (Kimura et al., 2011) suggesting that such compounds may nevertheless be useful. Fractions 4 and 5 from T091 neither showed decrease in hemolysis activity in a dose-dependent manner ($\beta_{fraction4} = 0.00$, $p = 0.46$, $\beta_{fraction5} = 0.00$, $p = 0.35$) but fractions 4 and 5 from T160-2 did ($\beta_{fraction4} = -0.013$, $p = 0.17$, $\beta_{fraction5} = -0.011$, $p = 0.003$) (Figure 3).

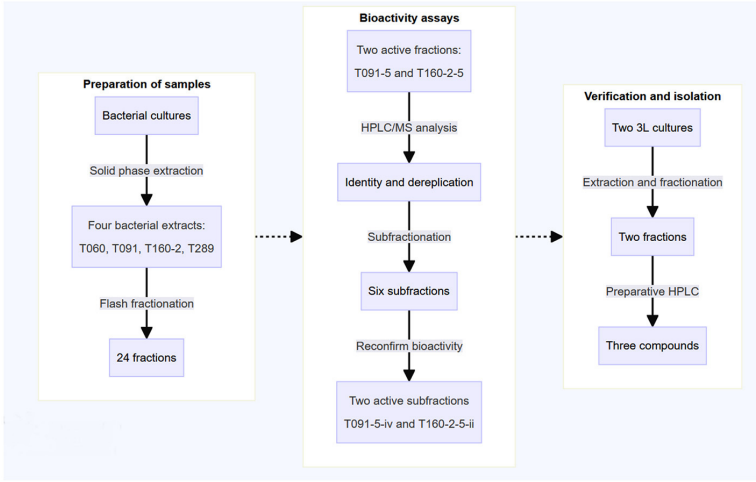


FIGURE 1
The schematic representation outlines the workflow employed in this study. Previously collected marine actinobacteria were cultured in 500 mL flasks in marine modified ISP2 media. Subsequently, the extracts underwent solid-phase extraction and fractionation using reversed-phase liquid chromatography, resulting in the generation of six fractions per sample (24 in total). The crude fractions were then screened for bioactivity in virulence-related *in vitro* assays, monitoring both the translocation of Tir and actin pedestal formation. The active fractions were also checked for their capacity to induce red blood cell hemolysis. Following this, the two active fractions were further fractionated and the activity of these subfractions was reconfirmed. Finally, the isolation and analysis of the active compounds was performed using RP-HPLC-MS via mass triggered fractionation.

3.4 Fraction 5 from the *Rhodococcus* sp. T160-2 prevents formation of microcolonies and the growth of EPEC

Bacterial virulence can also be inhibited, via non-specific mechanisms, i.e., generalized toxicity, such as that caused by growth inhibiting antibiotics. Inspection of the images revealed normal looking microcolonies when treated with fraction 5 from T091, suggesting that this was not the case (Figure 4A). Antibiotics typically cause the abolishment of microcolonies in the FAS-HCS assay and then very few adherent bacteria are visible on the cells as individual bacteria (no microcolonies), so this can be used as an indirect measure of toxicity. Fraction 5 from T160-2, in contrast, did decrease the size of microcolonies severely (Figure 4A; Supplementary Figure S2). These images also suggest that fraction T091-5 is not preventing adhesion entirely either, as there are clearly bacteria and microcolonies in the images (Figure 4A). Therefore, all fractions showing activity were also assessed for growth inhibition of EPEC in broth microdilution assays. Fraction 5 from T091 did not inhibit growth in this assay at concentrations up to 100 $\mu g\ mL^{-1}$, whereas fraction 5 from T-160-2 did (Figure 4B).

3.5 Individual subfractions of the crude FLASH fractions explain the bioactivities

Because the flash fractions contain a several compounds, further subfractionation (refractionation) of these primary fractions was performed using HPLC in order to reconfirm the activities and to get an improved understanding of the specific compounds involved. These subfractions were retested in the assays and then subjected to mass spectrometry to investigate their individual constituents. Following this, the individual molecules of the fractions were purified and tested again in the assays to reconfirm the active ones.

From T091-5, four subfractions, supposedly representing the major constituents (i, ii, iii, iv) were isolated using MS-coupled preparative HPLC equipped with a RP18 column, and then re-examined in the FAS-assay by dissolving into 2.5% DMSO with MQ, and applied to the cells dissolved in 35 µL of MEM. From these one (iv) decreased the average proportion of colonies with pedestals (0.63) compared to the no treatment (0.84) condition of the entire fraction, whilst the rest seemed to have lesser or no effect. Aurodox had a slightly lower mean effect of 0.56. Inspection of the images indicated similar decreases in actin condensates underneath the colonies between iv and aurodox (Figure 5A). Similarly, fraction T160-2-5 was subfractionated into two subfractions (i and ii), of which one (ii) showed similar growth inhibitory activity, whereas the other one (i) did not show any kind of growth inhibition (Figure 5B).

3.6 Isolation of compounds

Two subfractions were therefore considered bioactive. From strain T091 two compounds were isolated. The 3 L culture of T091 yielded 3.68 g of extract that was fractionated into six fractions.

10.3389/fmicb.2024.1432475

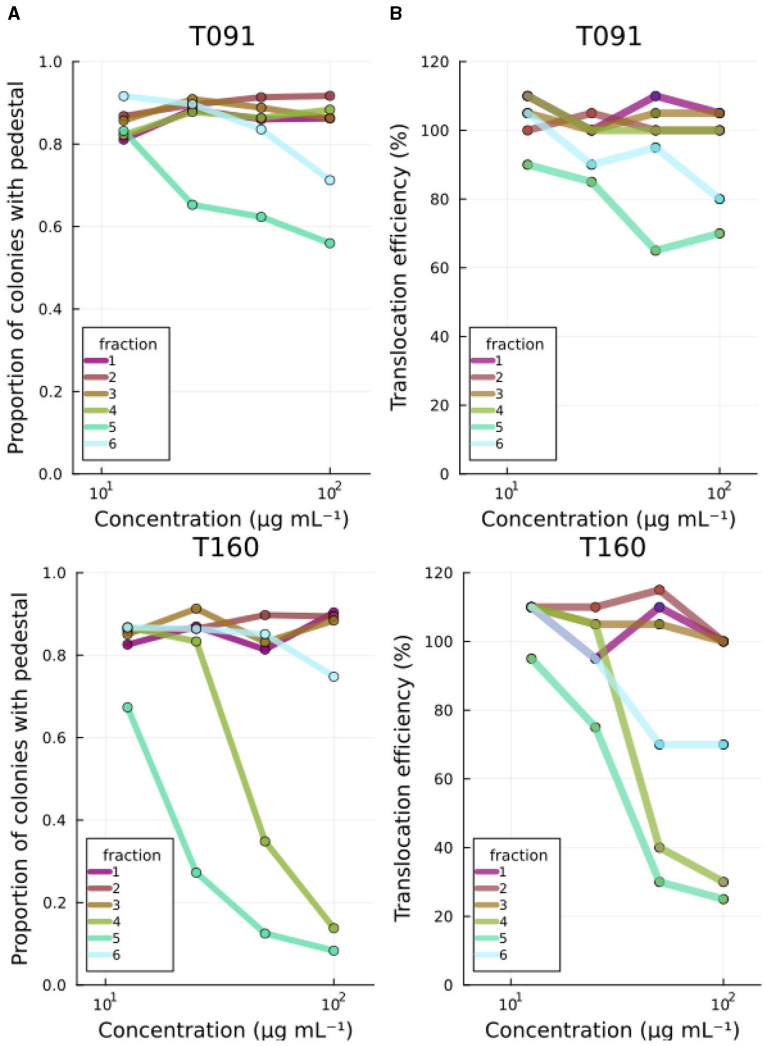


FIGURE 2
Primary screening results. **(A)** Fractions 5 from T091 and T160-2 decrease both the number of EPEC colonies with actin pedestals and **(B)** the translocation efficiency of the translocated intimin receptor in a concentration-dependent manner. The images are segmented by microcolonies, and the actin condensation under these is analyzed from the equivalent area in the phalloidin channel from the cells by segmenting inside of the first segments from the bacterial signal, thus creating a readout of proportion of microcolonies with detectable actin condensation beneath. For translocation assays, the readout is based on the FRET signal from the LifeBLAzer CCF4-AM dye and normalized to No treatment. The primary screening was performed at four concentrations (100, 50, 25, and 12.5 μg mL^{-1}). Fractions from the other strains had no effect (see Supplementary Figure S1).

Fraction 5 (321 mg) and 6 (108 mg) contained compounds **1** and **2**. In the first round of purification using the setup described under Section 2.5, the two molecules were isolated using a Sunfire RP18 column and a gradient from 25 to 100% (v/v) B in 15 min, the quadrupole was recording m/z from 200 to 800 in ESI+ and the signals m/z 782.5 and m/z 769.5 (low resolution MS) were set to trigger the collection of the eluents. The retention times were 8.36 min for **1** (yielding 2.4 mg) and 8.74 min for **2** (yielding 5.3 mg). In a second round of purification **1** was further purified using a Fluoro-Phenyl column and a gradient from 25 to 100%B (v/v) in 15 min, the retention time of 1 within that condition was 7.19 min. **2** was purified in a second purification step using a Fluoro-Phenyl column and a gradient from 25 to 75% (v/v) B in 14 min, the fraction from the first purification still contained traces of **1**. Compounds **1** and **2** were therefore collected at retention times of 7.87 and 8.25 min, respectively. The isolations

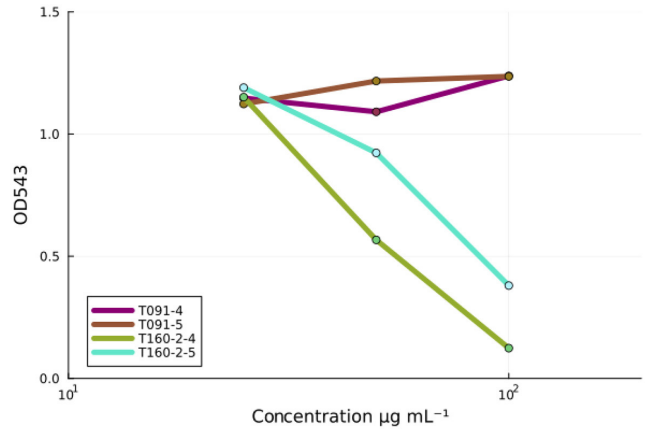


FIGURE 3
The inhibition of EPEC-induced red blood cell hemolysis by fractions. Fractions (4 and 5) from the strain T091 do not prevent EPEC induced hemolysis of sheep red blood cells at any concentrations, whereas fractions (4 and 5) from the strain T160-2 inhibit red blood cell hemolysis in a concentration dependent manner (100, 50, and 25 µg mL^{-1}). Linear models fit to the data: T091 $\beta_{fraction4} = -0.013$, $p = 0.17$, $\beta_{fraction5} = -0.011$, $p = 0.003$ and T160-2 ($\beta_{fraction4} = 0.00$, $p = 0.46$, $\beta_{fraction5} = 0.00$, $p = 0.35$). These results suggest that the observed activity in the screening assays is not due to the inhibition of T3SS-based injection, as compounds, such as aurodox, that do inhibit the expression of the T3SS injection needle, are known to inhibit hemolysis.

were executed by multiple injections and pooling of the respective fractions. The final yields after pooling and drying were **1**: 1.2 mg of red-brown wax-like compound and **2**: 0.9 mg of pale-brown wax-like compound. However, upon attempting to isolate more of the compound, isolation of **3** from 3 L culture of T160, yielding 2.01 g of crude extract was not successful, no visible or weighable amount of compound could be collected.

3.7 Identification and de-replication of compounds

As the individual compounds causing the bioactivities were recognized, we succeeded to analyze their structure and perform dereplication. The compound (from T091-5) that appeared to be changing the properties of the microculture and adherence of the bacteria was demonstrated to be a phospholipid. HPLC-HR-IMS-MS analysis revealed two potentially bioactive compounds in the active subfraction of T091, which are potential phospholipid-like compounds according to their elemental composition, and one potentially bioactive compound from the subfraction of T160-2-5 (see Table 2).

4 Discussion

Natural product mixtures derived from sources such as plants, bacteria, or animals, comprise a diverse collection of major and minor compounds, and therefore the analysis of them is a more challenging endeavor than the evaluation of individual pure compounds. The complex composition of these extracts requires prefractionation techniques to reduce complexity (Appleton et al., 2007; Tu et al., 2010) and certain constituents within these extracts, such as fluorophores, chromophores, or compounds harboring pan assay interference (PAINS), can directly influence assay outcomes (Bisson et al., 2016; Bolz et al., 2021). The reduced complexity of flash fractions compared to crude extracts and the possibility to compare active with "neighboring" inactive fractions eases the de-replication of active fractions significantly. In our experience, fractionation increases the relative concentration of potentially active compounds (e.g., in relation to media components or inactive metabolites), which then enables their detection using *in vitro* screening. Additionally, employing multiple distinct assays alongside quantitative and qualitative assessment based on raw images enables us to address these complexities effectively. Imaging and other high-content methods are notably less susceptible to interference on detection signals, as the artifacts can typically be directly recognized from the images by the operator or well-designed quality control in the analysis pipeline. For example, surfactants, particularly rhamnolipids, have recurrently exhibited bioactivity when screening extract fractions from marine bacteria, demonstrating efficacy in both antibacterial and anticancer screenings within our laboratory (Schneider et al., 2019; Kristoffersen et al., 2021). In this study, we noticed very little, if any, interference in the measurements based on optical density, despite some of the fractions being somewhat dark in appearance. It is worth noting that the widely used optical density measuring hemolysis assay has been modified into an imaging-based method, wherein problematic fractions could be reevaluated in case of uncertainty (Knutton et al., 2002). Nonetheless, to our knowledge, this study marks the first utilization of the Tir-translocation and FAS-assays for bioassay-guided discovery from

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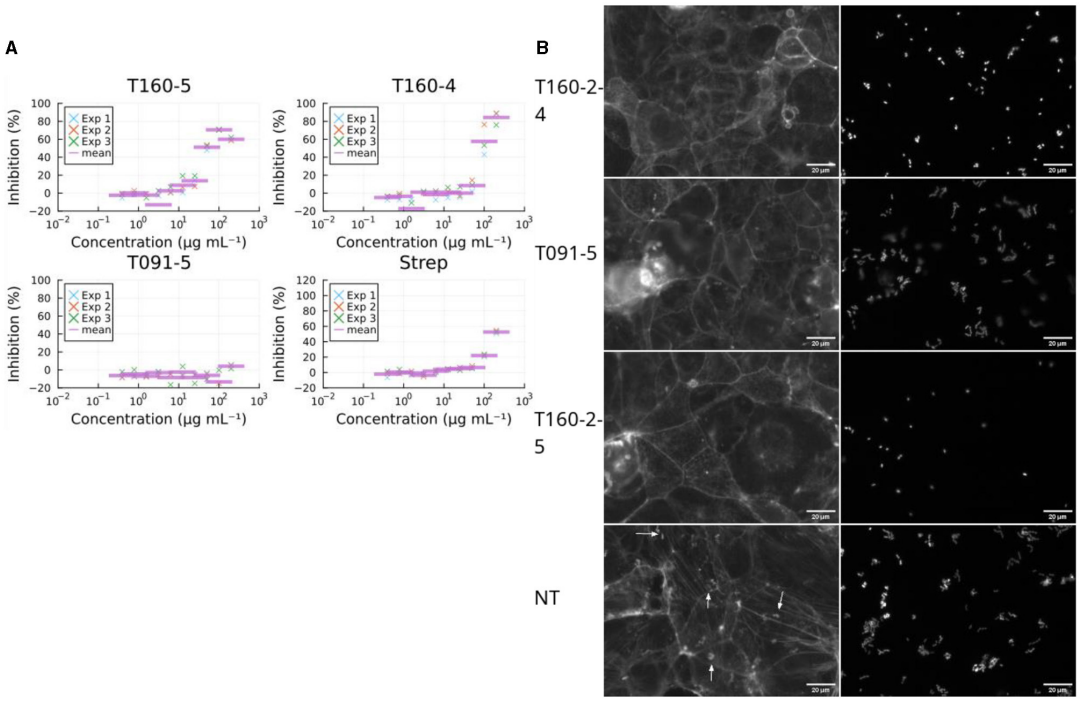


FIGURE 4
Effects on growth. **(A)** Fraction T091-5 did not inhibit growth in the broth microdilution assay (at 6 h post infection, three biological replicates marked by "×" and their arithmetic mean with a purple bar). This supports the view that the decrease in virulence caused by the compounds in fraction 5 of T091 do not achieve this via a bactericidal or growth inhibiting mechanism of action, whereas the compounds of fractions 4 and 5 from T160-2 are likely growth inhibitory. Streptomycin is shown for comparison, the strain (E2348/69) carries resistance genes for streptomycin (*strA* and *strB*), in contrast T091-5 does not show inhibition at any concentration. The results are normalized to the no treatment condition and 4 μg mL^{-1} gentamicin. **(B)** Fractions also change microcolony morphology in the coinfection model. In the no treatment condition (Size mean = 102) the bacteria form normal microcolonies, where multiple bacteria adhere in a pattern called "localized adherence", whereas in the growth inhibiting conditions smaller colonies or even individual bacteria are only present. Mean sizes at 100 μg ml^{-1} T160-2 f4 = 54.7, T160-2 f5 = 59.7 and T091 f5 = 137. Arrows indicate actin condensation foci, the scale bar is 20 μm in size.

TABLE 2 **Elemental compositions calculated from ESI-HR-MS2 data.**

Compound	Observed *m/z* and observed RT	Calcd. *m/z*	Mass error:	Observed CCS	Formula:	Final yield
1	782.46051 $[M + H]^+$ 7.01 min	782.46082	0.396186 ppm	279.99 Å^2	$C_{41}H_{68}NO_{11}P$	1.2 mg
2	796.47660 $[M + H]^+$ 7.39 min	796.47647	0.163219 ppm	281.52 Å^2	$C_{42}H_{70}NO_{11}P$	0.9 mg
3	316.10369 $[M + H]^+$ 3.7 min	316.10458	2.815524 ppm	160.04 Å^2	$C_{14}H_{13}N_5O_4$	–

bacterial extract fractions, providing evidence for the applicability of these assays as valuable tools for the bioprospecting of specific inhibitors of virulence.

Many antimicrobial and virulence inhibiting compounds have previously been discovered in natural products of especially microbial origin. For example, one of the first type three secretion system inhibitors ever discovered was the glycolipid caminoside A isolated from extracts of the marine sponge *Caminus sphaeroconia* (Linington et al., 2002). The compound was discovered using an ELISA-based high-throughput assay to screen a large (20,000 compounds) library monitoring virulence protein secretion via the pore forming EPEC secreted translocator EspB (Gauthier et al., 2005). Caminoside A decreased the secretion of the EspB from EPEC culture into the supernatant without effect on the secretion of other proteins nor bacterial growth. Guadinomines, the most potent known T3SS inhibitors, were similarly discovered using EPEC-mediated red blood cell hemolysis to screen natural product extracts (of *Streptomyces* sp. K01-0509) (Iwatsuki et al., 2008). In another study, this same screening assay methodology was used to discover polyketides generated by *Streptomycetes* that appear to particularly inhibit virulence molecule expression in EPEC (Kimura et al., 2011). It was later demonstrated that the most potent of these, aurodox, down-regulates virulence genes on a pathogenicity island (Locus of enterocyte effacement) by affecting

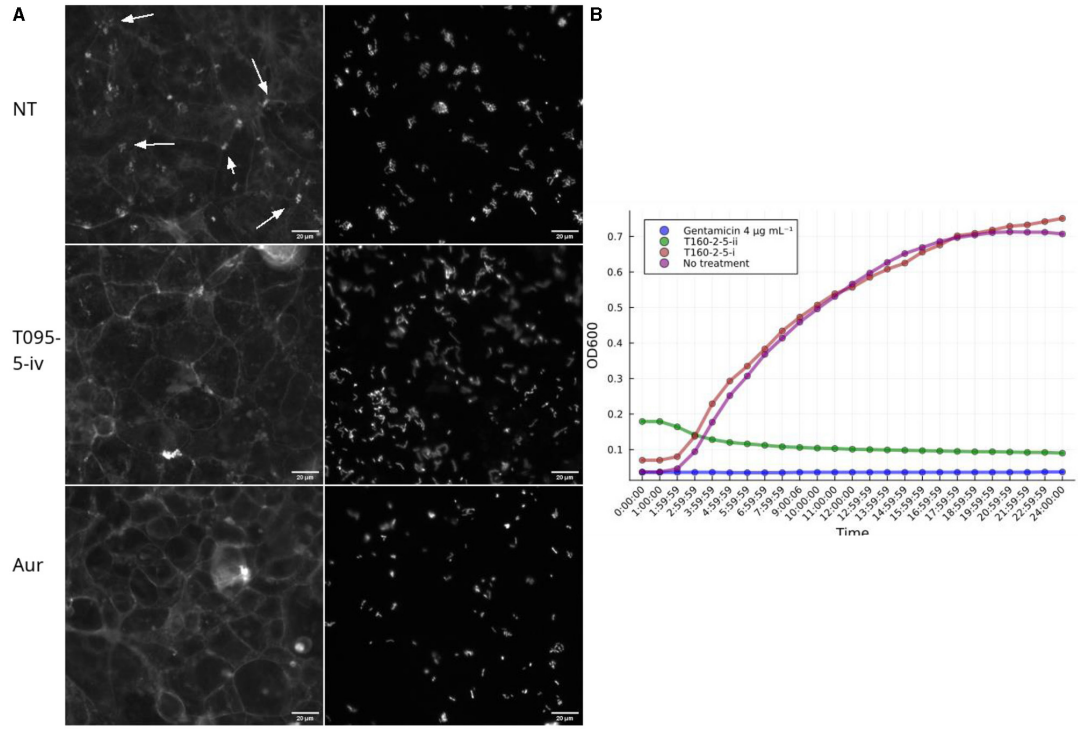


FIGURE 5
Subfractions demonstrate a similar biological activity as the full fractions in the screening assays. **(A)** Example images of pedestal inhibition. Each picture depicts a Caco-2 cell monolayer that is stained using phalloidin-Alexafluor488 and on the right EPEC-mCherry. Subfraction iv from T091-5 shows decrease in actin condensations, whereas in the no treatment (NT) condition there are multiple pedestals apparent as bright colony-sized spots. Aurodox (aur), a T3SS inhibitor that prevents actin pedestals in EPEC infections. **(B)** The two subfractions (i and ii) isolated from the fifth flash fraction of T160-2 had opposite effects on growth kinetics of EPEC, so that T160-2-5-ii is inhibitory similar to 4 µg mL^{-1} gentamicin, whereas T160-2-i is similar to no treatment.

an upstream regulator, Ler (McHugh et al., 2018). Recently, EPEC Tir translocation inhibitors were discovered from bacterial metabolite collections utilizing a high-throughput translocation screening assay (Mühlen et al., 2021). The active compounds appear to not affect the expression of EPEC virulence genes, but nevertheless decrease the translocation of effectors into the host cell by as of yet unknown means.

We studied the effects of the marine actinobacterial extracts on EPEC virulence and growth. Fraction 5 from the strain T091 inhibited EPEC caused actin condensation and the translation of the translocated intimin receptor. Analysis of the images and data reduced from them on bacterial counts suggests that subfraction T091-5-iv is not eradicating the bacteria, and the fact that this fraction does not show inhibition of growth suggest that it does not have antibiotic activity. Interestingly, the subfraction neither prevented red blood hemolysis, a phenomenon believed to be caused by the pore forming capacity of translocators such as EspB/EspD which are a part of the T3SS injection needle. Antibiotic compounds typically prevent EPEC-induced hemolysis, as do compounds that down-regulate the genes from the Locus of enterocyte effacement pathogenicity island of EPEC. Such down-regulation could otherwise explain the decreased translocation and subsequent actin condensation. However, the compounds (**1**, **2**) in the subfraction (T091-5-iv) responsible for the activity are large (molecular weight around 700) phospholipids by composition and are unlikely to access intercellular compartments of the bacterial cells. Additionally, it is known that EPEC uses multiple pili and attachment molecules both to adhere to cells and to autoaggregate into microcolonies. Because it is known that adherence of typical EPEC strains—such as the EPEC E2348/69 used here—to cells and other EPEC individuals is largely mediated by a type 4 pilus, called the bundle forming pilus, and that one target of this pilus is cell wall phospholipids (Barnett Foster et al., 1999; Wu et al., 2004), it is possible that the compound acts by competing with the membrane-based ligand, thus decreasing the adherence of the pathogen to the cells. For example, naturally occurring EPEC strains that do not express the bundle forming pilus (BFP), for example due to not carrying the pEAF plasmid, do not display localized adherence, but typically adhere in a diffuse pattern (Rocha et al., 2011). The main target of BFP is believed to be phosphatidylethanolamine,

which is the second most abundant phospholipid present in the plasma membrane of eukaryotes (Barnett Foster et al., 1999). On the other hand, one would expect to see smaller microcolonies if this were the case. Further investigation is needed to uncover the exact mechanism of action in more detail.

In addition, we discovered that compound **3** from the subfraction T160-2-5-ii from the *Rhodococcus* T160-2 was able to decrease actin condensation and Tir translocation. This, however, showed clear signs of growth inhibition in EPEC both in the images from the infection models, but also in broth microdilution assays. Therefore, these effects are clearly caused by a decrease in viable organisms and not specific virulence related mechanisms. This compound was tentatively identified to be the cause of this EPEC growth inhibiting effect and is under further investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

TP: Conceptualization, Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. YS: Investigation, Methodology, Writing – review & editing. TR: Conceptualization, Writing – review & editing. JA: Conceptualization, Writing – review & editing. PT: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1432475/full#supplementary-material

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