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Receptor-mediated host cell targeting by *Staphylococcus aureus* pore-forming cytotoxins

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Cover: The *Staphylococcus aureus* pore-forming cytotoxins have been extensively studied for more than one hundred years; however, the mechanism by which these toxins target specific cell types and/or exhibit species specificity was largely unknown until recently. On pages 21–27, DuMont and Torres discuss the identification of several nonredundant, proteinaceous surface receptors utilized by the *S. aureus* cytotoxins to target and kill host cells. The cover shows an artistic interpretation of the battle between *S. aureus* and host polymorphonuclear cells (PMNs), specifically highlighting the use of cellular receptors for the targeted lysis of PMNs by staphylococcal cytotoxins. A PMN (in teal) on the left attempts to phagocytose and kill *S. aureus* (yellow spheres), while *S. aureus* combats the PMNs by secreting numerous cytotoxins (various colors) into the extracellular milieu. The toxin monomers come in contact with PMNs via specific cell surface receptors (various colors) and oligomerize to form pores in the PMN membrane as seen on the most forefront cell. The PMN in the top right corner is undergoing osmotic lysis due to extensive membrane damage caused by cytotoxin pores. Image courtesy Ashley DuMont.

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‘Globish-lization’: a worldwide non-native English problem or a chance to evolve?

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In the October issue of *Trends in Microbiology*, Norris [1] and Tychinin and Kamnev [2] discuss the worldwide problem of writing a scientific paper at a level that is ‘adequate’ especially for non-native English speakers. Many of us, when reviewing papers or projects of our peers, or writing our own papers, are aware of the difficulty in communicating science and the results of scientific research in a manner that is both appealing and grammatically correct. The stylistic differences between American and British English pose an additional problem for non-native English speakers when formatting or editing a paper for a journal, as well as the differences in punctuation and spelling.

A ‘radical solution’, which Norris aptly illustrated, is the adoption of ‘Globish English’. This term was proposed by Jean-Paul Nerriere, a retired vice-president of IBM (US) (http://www.theaustralian.com.au/news/world/globish-now-the-lingua-franca-of-world-travellers/story-e6frg6so-1111112672356), to designate a ‘language’ that has a vocabulary of only 1500 English words and a subset of standard English grammar [http://en.wikipedia.org/wiki/Globish_(Nerriere)]. This simplified language, which was designed for the non-native English speaking community to communicate science, may conceal many problems that are worse than their solutions. I agree that simplified sentences are beneficial for clarity and understanding, but Globish English *per se* cannot be ‘the’ solution. Consider the case of a clinical geneticist who is studying a rare variant of a particular disease. A tremendous effort by that doctor would be required simply to explain to the community that he used a ‘next generation sequencing’ approach to find the ‘variant’ that determined the particular disease or that he ‘validated’ the result by means of a ‘digital’ PCR. The Globish English dictionary includes ‘real-time experiment’, but nothing more complex. For the microbiological community, ‘metagenomic’ approaches would not be possible and the ‘microbiome’ would continue to be a ‘perfect stranger’. If you are a chemist who works in the ‘nanotechnology’ or ‘biotechnology’ field, you will need to find alternative terms for ‘nanoparticles’, ‘carbon nanotubes’, ‘reactors’ and even drug delivery ‘vectors’. As an exercise, try to determine how removed your sentences are from perfect Globish language following the link shown here (http://www.globish.com/?page=globish_scanner). The dedicated scanner will alert you immediately to the level of difficulty that you will face in preparing a scientific paper for a ‘non-native Globish’ community. The letter you are reading has a length of 605 words and contains 108 (18%) non-Globish words.

I think that ‘Globish for Dummies’ is unlikely to become a bestselling book if it is intended to teach students how to write about science. Instead, it may mark the beginning of a dialogue within the scientific community on standardizing the way in which papers are written. We could attempt to define a sort of ‘linguistic’ Minimum Information Standards (http://en.wikipedia.org/wiki/Minimum_Information_Standards) as has been done already for microarrays (MIAME) [3], for quantitative real-time PCR experiments (MIQE) [4], and for digital PCR (dMIQE) [5]. This would provide a sort of guide or common code to use in reporting science. Protecting English (or any other languages) from its own success, as Norris has suggested, is not a solution. Science and technology inevitably evolve and, therefore, so does their language. We should learn from evolution in nature and let our language and vocabulary evolve in order that we become modern science writers. The ‘overworked class’ of ‘screenagers’, as scientists inevitably are, should be aware of buzzwords and other neologisms that have been coined in recent years (http://www.learn-english-today.com/new-words/new-words-in-english1.html) and consider using them in their papers. Our language must evolve and so should we evolve. Limiting scientific writing to using a vocabulary of only 1500 words is like reporting a great piece of research by means of a letter to the editor.

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Seeing is believing: what experiments with microbes reveal about evolution

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Darwin's theory of natural selection is among the most powerful ideas in science, yet evolutionary ideas remain challenged to this day. This is in part because evolution often cannot be directly observed. Simple experiments with microbes can change that by enabling direct observation of evolutionary processes.

Evolution in a Petri dish

We recently carried out an evolutionary experiment where we made the bacterium *Pseudomonas aeruginosa* go through consecutive rounds of swarming. Swarming is a form of collective motility where *P. aeruginosa* colonies travel over soft surfaces in a branched pattern. Over the course of this experiment the colonies evolved a new morphology called hyperswarming where they cover the entire plate rather than branch out. The genome of *P. aeruginosa* has 6 million nucleotides and finding the causative mutations, which is like finding a needle in a haystack, could have been very costly even 5 years ago. These days, next generation sequencing and bioinformatics methods are more accessible and it was relatively easy to find the mutations. We used classical approaches, gene complementation, and transcription expression assays to confirm the sequencing results and understand the molecular mechanisms involved. Hyperswarming was caused by single point mutations in the gene *fleN*, which turned the normally monoflagellated *P. aeruginosa* into a polar multiflagellate and improved swarming [1].

These results shed new light on the genetics of flagella. Before our experiment it was known that *fleN* regulates flagellar number but *fleN* knockout mutants cannot swim, so finding hyperswarmer mutations in this gene was unexpected. Furthermore, the same mutations that enabled the bacteria to swarm better also impaired their ability to form biofilms, a finding that can potentially be exploited in anti-biofilm therapies [2].

In addition to molecular insights, the hyperswarming experiment has two features relevant for evolution. First, the experiment is surprisingly reproducible. All hyperswarmers evolved in independent experiments had point mutations in *fleN*. This was difficult to anticipate because swarming in *P. aeruginosa* involves many genes [3] and our finding supports that evolution can be, under certain conditions, predictable. Second, the mutations produce changes at the cellular level that lead to better colony spreading. Hyperswarming is easy to explain in pictures just like a textbook example of experimental evolution (Figure 1).

Our study is, of course, not the first to adapt microbiology assays for experimental evolution (see a recent review [4]). Microbes make great models owing to short generation times and huge population numbers, and evolution can itself be used as a tool for microbial genetics [5]. The same integration of molecular and evolutionary methods is within reach for every experimental microbiologist and can lead to powerful demonstrations of evolution. Simple experimental evolution with harmless bacteria, such as the non-pathogenic relative of *P. aeruginosa*, *Pseudomonas fluorescens* [6], can even be adapted for educational purposes [7].

In order to do experimental evolution it helps to consider a straightforward definition: evolution is the change of gene frequency in a gene pool. The definition derives from the work of Fisher, Haldane, and Wright, who integrated Mendelian heredity and Darwin's natural selection into population genetics [8]. Gene frequencies can change through four mechanisms: mutation, natural selection, genetic drift, and migration. Mutation and natural selection readily come to mind when thinking about evolution. Mutation, a change in the heritable material, is a process that generates genetic diversity. Selection acts on that diversity, favoring alleles with increased reproductive success. Drift and migration may be less discussed but are equally important. We present examples of all four processes below. Any evolutionary experiment can be interpreted according to these four elementary mechanisms, as long as the gene pool and a timespan of evolution are properly defined. Experiments are often a combination of some or all of these four processes. Here are some notable examples.

Mutation and selection

In 1943, Luria and Delbrück published their famous fluctuation experiment [9]. They asked whether the resistance to bacteriophage in *Escherichia coli* was induced by the presence of phage or if instead it was due to random mutations occurring prior to phage exposure. Plating *E. coli* on agar plates with phage and counting colonies followed by sound theoretical analysis led to the conclusion that resistance came from prior mutations. This was the first direct demonstration of the random nature of mutation [10]. Luria

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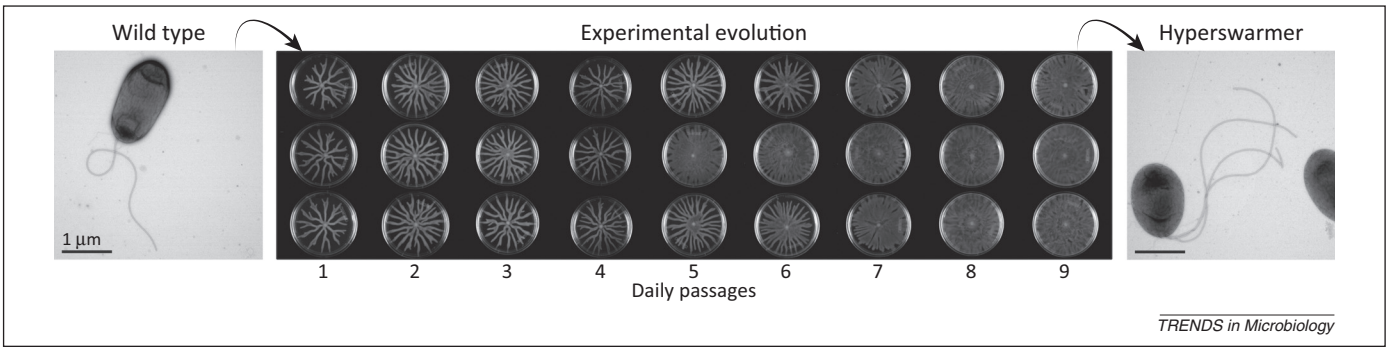


Figure 1. The hyperswarming experiment. Three independent lineages were started from the same ancestral *Pseudomonas aeruginosa* (left panel) and passaged daily in swarming assays in Petri dishes [1]. Over the 9-day experiment, the colony morphology evolved from a branched pattern to a round shape covering most of the Petri dish, a phenotype we call hyperswarming (center panel). In replicate experiments, hyperswarming was always caused by point mutations in the same gene, the flagellar synthesis regulator *fleN*, which make the bacteria change from being monoflagellated to being multiflagellated (right panel).

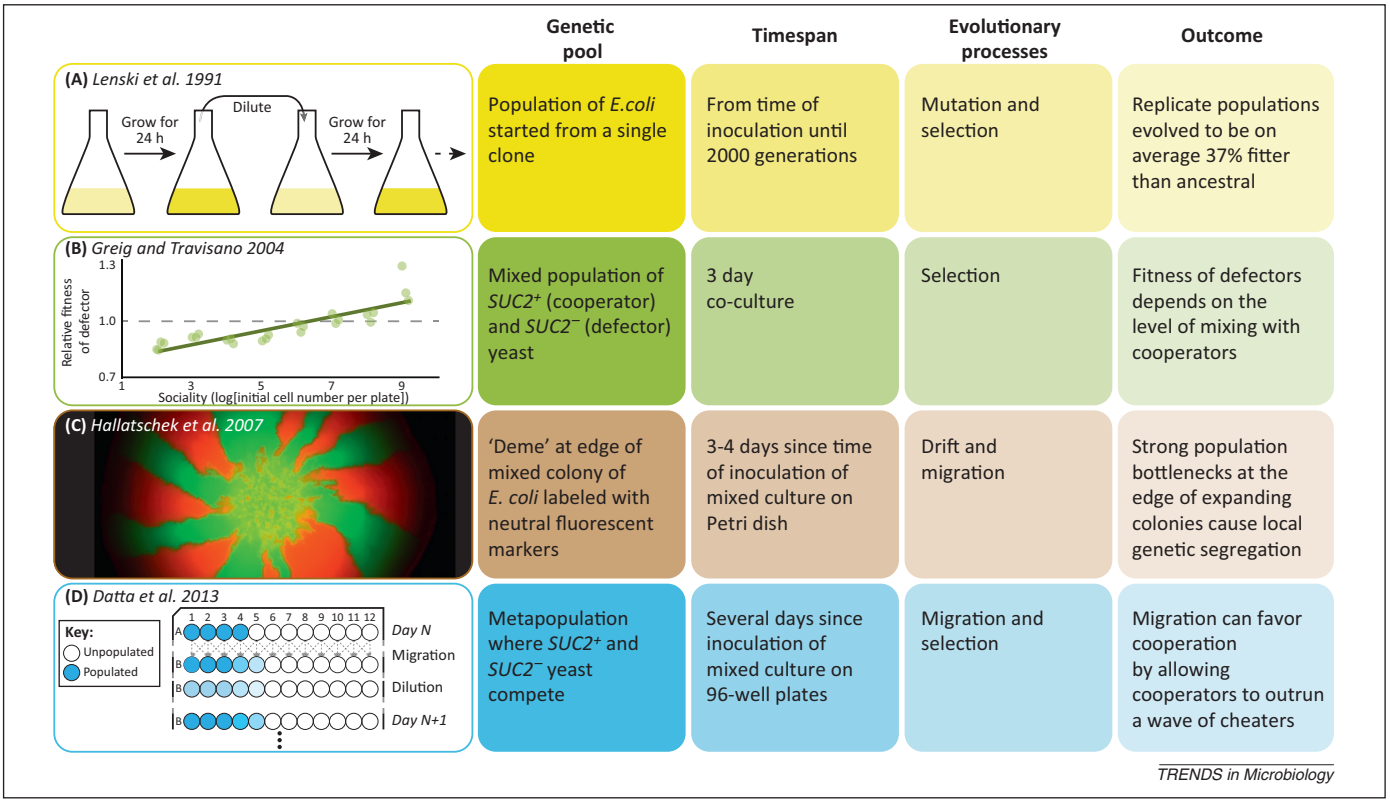


Figure 2. Microbial models of evolution. **(A)** The long-term evolutionary experiment of Lenski and colleagues consists of the daily passaging of parallel populations of *Escherichia coli* [11]. **(B)** Investigation of frequency-dependent selection by mixing two strains and measuring their numbers before and after competitions. Plot adapted from the experiment of Greig and Travisano using the yeast *SUC2* model [13]. **(C)** Genetic drift is observed in bacterial colonies by mixing two variants of *E. coli* differing only in the expression of a fluorescent marker (shown in green or red) [14]. Picture courtesy of Oskar Hallatschek. **(D)** An experimental analog of the 'stepping-stone' model used to study the role of migration in the evolution of cooperative yeast [15].

later was the PhD advisor of Watson who, with Crick, identified the structure of the heritable material, DNA. The work of Luria and Delbrück launched the discipline of molecular biology and they received the Nobel Prize in 1969.

In 1991, Richard Lenski and colleagues published the first results on a long-term experimental evolution [11]. The experiment consisted of daily passages of 12 populations of *E. coli* started from a single clonal ancestor (Figure 2A). The populations evolved over 2000 generations, becoming on average 37% fitter. Consistent with the randomness of mutations, the populations evolved at different rates. The long-term experiment of Lenski has now been running for more than 25 years and is rapidly approaching 60 000 *E. coli* generations. It has inspired many studies, of which the hyperswarming experiment is but one example [1].

Frequency-dependent selection

In some cases, the fitness of an organism depends on what other organisms in the same population are doing. For example, organisms that carry out cooperative actions face a problem when others in the same population are not cooperative. If selection favors selfishness how can cooperators resist exploitation? How cooperative behaviors evolve is a fundamental issue in evolution [12] that has become tractable through microbial experiments. *Saccharomyces cerevisiae* requires the gene *SUC2* to break down

sucrose into fructose and glucose. Strains lacking *SUC2* cannot grow on sucrose. In a co-culture experiment $SUC2^-$ mutants exploit $SUC2^+$ strains because they can metabolize free glucose and fructose without contributing to sucrose digestion. Thus, selection for *SUC2* is frequency-dependent (Figure 2B), which may explain why the SUC family of genes is polymorphic [13].

Drift and migration

Although genetic drift is evolutionarily important, it is not necessarily intuitive. Fortunately, drift can be observed in simple experiments using neutrally labeled *E. coli* strains. When growing a two-color mix of *E. coli* spotted on a Petri dish for 3–4 days, it will look normal under regular light. Fluorescence imaging reveals unmixing of the two strains in beautiful radial sectors (Figure 2C). The explanation for sectoring is that as the colony grows, the center becomes nutrient-depleted and only bacteria at the edge can reproduce. A stochastic process determines which bacteria make it to the next generation [14]. The sectors emerge from genetic drift, a random change in local gene frequency.

Migration and selection

Most gene pools are not closed systems and gene frequency can change because of migration into and out of the system. Sometimes migration can lead to a paradoxical selection for deleterious mutations. Here, too, microbial experiments can provide powerful insights; 96-well microtiter plates can be turned into analogs of the 'stepping stone' model, a classical model to describe dynamics of subdivided populations in population genetics, and used to investigate how migration shapes evolution (Figure 2D). Again using the yeast *SUC2* system, it was recently shown that migration can favor cooperation in an expanding population through the deterministic enrichment of cooperators at the expansion front, and by allowing cooperators to outrun an invading wave of cheaters [15].

Concluding remarks

Evolution is key to understanding all life; still, key evolutionary ideas remain challenged to this day. This is in part because the evolutionary process is often not directly observed but, rather, it is inferred by comparing present-day species, fossil records, and, more recently, DNA sequences. Experiments with microbes allow the observation of evolutionary change on short absolute time scales. Evolutionary biologists that study organisms that can only be seen by the naked eye can miss the power of microbial models [10]. Microbiologists who focus on molecular mechanisms have established many assays that can be used both to study and to communicate evolution. With exciting advances in experimental evolution and new technology, such as affordable whole genome sequencing, it is time that evolutionary biologists and microbiologists come together towards a better understanding of evolution.

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Handle energy resources with care

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While politicians discuss rules to improve the efficacy of global energy consumption and to limit environmental pollution, the mammalian body has numerous mechanisms to efficiently use nutrient resources and appropriately extract energy. A new mechanism has recently been identified by the group of Fredrik Bäckhed in *Cell Host & Microbe*.

Glucagon-like peptide-1 (GLP-1) is a gut hormone derived from the preproglucagon precursor protein. The bioactive form (7-37) is generated by selective cleavage of proglucagon. GLP-1 is produced by enteroendocrine L cells found in the intestinal tract but also by pancreatic α cells and brain tissue. Secretion is induced by oral nutrient intake and luminal exposure of L cells with sugars, amino acids and lipids as well as endogenous neuronal and hormonal stimuli. Postprandial secretion of GLP-1 potentiates glucose-induced insulin secretion in the pancreas (the so-called incretin effect) and causes decreased appetite and reduced stomach emptying. This 'ileal brake' is thought to regulate the postprandial glucose absorption via both afferent and efferent vagal innervation (reviewed in [1]). Whereas sugar and amino acids reach the highest concentration in the proximal small intestine shortly after nutrient intake, stimulation of L cells with short-chain fatty acids (SCFAs) is thought to occur mainly in the distal gut [2].

Fredrik Bäckhed had previously characterized the contribution of the enteric microbiota to nutrient digestion and energy storage in a comparative approach using germ-free and conventional (i.e., bacterially colonized) mice [3]. Based on his previous results, Bäckhed and colleagues hypothesized that the lack of microbiota-mediated fermentative activity of complex plant polysaccharides in the colon of germ-free mice should reduce concentrations of SCFAs and thus lead to low GLP-1 levels. Surprisingly, however, they found that germ-free mice have elevated GLP-1 levels in portal vein plasma, enhanced proglucagon mRNA expression levels in gut tissue, and increased numbers of GPI-1 producing L cells in the colon and caecum tissue and they started to investigate this further [4].

Similar to germ-free mice, reduction of the microbiota in conventional animals by antibiotic treatment decreased SCFAs and enhanced proglucagon expression. Conversely, colonization of germ-free mice with a complex microbiota reversed the phenotype over time. Of note, monocolonization with the potent fermenter *Bacteroides thetaiotaomicron* had a similar effect whereas colonization with *Escherichia coli*, unable to efficiently digest complex polysaccharides, did not show this effect. The critical role of SCFAs for colonic GFP-1 regulation was directly demonstrated by oral administration of tributyrin or high fat diet (HFD) to germ-free mice, which lowered proglucagon mRNA levels similar to *ex vivo* exposure of colon tissue to acetate, propionate and butyrate. Surprisingly, elevated GLP-1 levels in germ-free mice did not significantly influence glucose tolerance in contrast to the insulin-inducing effect of postprandial GLP-1 secretion. Instead, Wichmann *et al.* identified GLP-1-mediated reduction of small intestinal transit, which was reverted after feeding the mice a HFD or administering the GLP-1 inhibitor exendin 9-39 as well as colonization with a complex microbiota or *B. thetaiotaomicron* but not with *E. coli*. Reduced transit was also found in antibiotic-treated conventional but not *Glp-1* receptor knockout animals [4].

According to the new findings, low colonic energy availability indicated by low SCFA influences local GLP-1 secretion both by increasing the number of cecal and colonic L cells and proglucagon mRNA expression. Enhanced GLP-1 in turn delays small intestinal transit in a GLP-1 receptor-dependent fashion to increase energy gain from luminal nutrients through improved extraction of energy containing metabolites. Beside longer exposure of nutrients to bacterial digestive enzymes, the extended transit time might also lead to a higher density of bacteria in the colon, again improving the metabolic activity. However, sufficient energy availability would reduce the resident time in the colon, reducing the risk of bacterial infection. The mechanism underlying reduced gut transit and its effect on energy harvest was not examined but it may be a direct effect of GLP-1 on the enteric nervous system (ENS). The respective receptor, GLP-1R has been found widely expressed in various tissues including peripheral nerves [2].

The discovered gut intrinsic GLP-1 effect appears to act independently of the known postprandial incretin effect of GLP-1. Most strikingly, lack of colonic SCFAs induces enhanced GLP-1 levels in fasting germ-free mice, whereas SCFAs were previously identified as a stimulating signal for postprandial GLP-1 secretion [5]. Also, whereas the GLP-1 mediated incretin effect is most potently stimulated by free fatty acids and sugars in addition to proteins and hormonal and neuronal signals [6], colonic GLP-1 secretion appears to be mediated mainly by SCFAs. Finally, enhanced GLP-1 levels in germ-free mice did not affect glucose tolerance or satiety but regulated small intestinal transit generating a 'colonic brake' (Figure 1).

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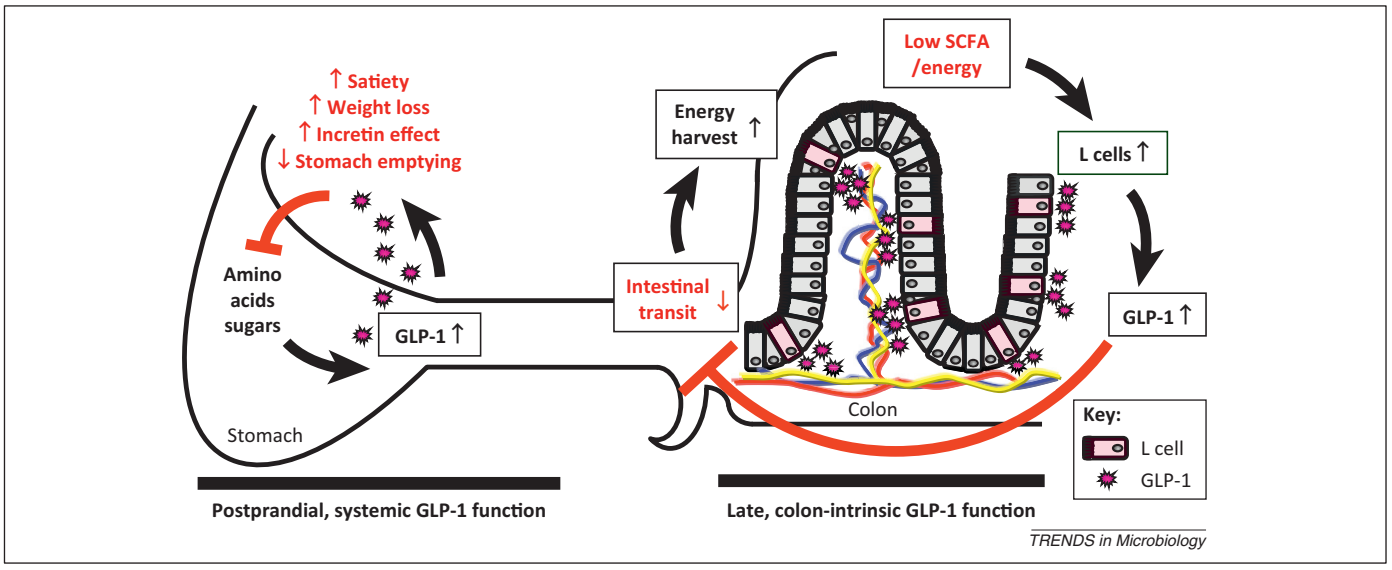


Figure 1. The Janus face of GLP-1. Postprandial availability of energy sources, such as amino acids or polysaccharides (sugars) leads to an upregulation of GLP-1 in the small intestine (left). Distribution of GLP-1 via the circulation causes the incretin effect and manifests as satiety and reduced stomach emptying. Conversely, low energy availability in the large intestine indicated by low concentrations of short-chain fatty acid (SCFA) such as seen in germ-free mice leads to increased numbers of enteroendocrine L cells (purple) associated with enhanced GLP-1 (stars) secretion. Colon-derived GLP-1 negatively regulates small intestinal transit and increases energy harvest from luminal nutrients.

Interestingly, the authors found a good correlation of SCFA availability, number of colonic L cells, proglucagon mRNA levels, and intestinal transit time but failed to demonstrate significant changes of GLP-1 peptide levels upon intervention. This might be due to the very short half-life of bioactive GLP-1 *in vivo* [7]. The short half-life of GLP-1 might also help to explain the two different scenarios. The described SCFA stimulation of cecum and colon-derived GLP-1 might act locally with little extraintestinal consequences, whereas postprandial GLP-1 secretion from the upper intestinal tract may enhance insulin secretion and induce additional systemic downstream effects. Analysis of bioactive GLP-1 peptide fragments present in gut tissue, portal vain blood and serum of germ-free mice, in addition to analysis of the local tissue distribution of the GLP-1 degrading enzyme di-peptidyl peptidase (DDP)-4 and the administration of DPP-4 inhibitors to germ-free mice might help to explain the different outcome.

The novel GLP-1 effect adds yet another level of complexity to the regulation of nutrient harvest. It was identified in germ-free mice and mice treated with high doses of antibiotics. It remains to be shown whether this gut intrinsic effect can also be observed in humans. Administration of GLP-1 agonists and DDP-4 antagonists has been extensively evaluated in type II diabetic patients. GLP-1 agonists have been reported to result in weight loss due to increased satiety and not enhanced energy harvest [8]. Treatment with GLP-1 agonists also can result in diarrhea, not obstipation but the underlying mechanism has not been defined. By contrast, enhanced GLP-1 levels and decreased intestinal transit time were observed in patients with anorexia nervosa [9]. Also administration of a GLP-1 agonist improved the clinical symptoms of short bowel syndrome [10]. The new results might thus help to develop new therapeutic strategies to improve chronic conditions of reduced caloric intake.

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To harm or not to harm? On the evolution and expression of virulence in group A streptococci

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Group A streptococci (GAS) cause three different types of infection (*sensu lato*) with distinct levels of virulence: asymptomatic colonization, superficial symptomatic infection, and invasive infection. To address why this pattern with several infection types has evolved, we combine mechanistic understanding from infection medicine with recent theory from evolutionary ecology. We propose that asymptomatic colonization and superficial symptomatic infection exploit different states of the host epithelium to maximize transmission between hosts in different epidemiological conditions, whereas the ability of the bacteria to cause invasive infection is a non-adaptive side effect of traits required for superficial symptomatic infection.

Group A streptococci and their types of infection

Humans host various bacteria. Most are commensals, some are pathogens, and some can be either. Pathogenic streptococci are an example of bacteria that can act as both commensals and pathogens, with GAS (*Streptococcus pyogenes*) as a prime example. A leading cause of death worldwide, GAS give rise to toxic shock syndrome, necrotizing fasciitis, and sepsis, but most clinical infections manifest as superficial and self-limiting pharyngitis and pyoderma [1,2]. In addition, GAS commensally colonize a considerable fraction of the healthy population [3]. The infections (*sensu lato*) caused by these bacteria can therefore be divided into three broad categories: the invasive (e.g., sepsis); the superficial symptomatic (e.g., pharyngitis); and commensal colonization. Infections of different types may occur in sequence or in isolation. The existence of different types of infection with distinct levels of virulence is not unique to GAS and other β-haemolytic streptococci, but is a general phenomenon that applies also to *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* [4–6].

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The phenotype of an infection is a result of interactions between the pathogen and its host, and the existence and relative frequency of different types of infection may therefore be explained by variation in either or both of these parties. Hosts differ in their propensity to acquire certain types of infection. For example, invasive GAS infections are associated with old age [7] and with preceding infection with varicella-zoster virus [8]. The relative frequency of different types of infection can therefore change with demography and healthcare practices, such as varicella vaccination. In addition, there is a host genetic component to the severity of invasive disease [9] and there is some indication that certain individuals are disproportionally likely to become asymptomatic carriers [10]. However, some GAS serotypes are biased towards a given infection type, which shows that the bacterium also plays a role in determining the type of infection. For example, the serotypes M1 and M12 are both able to cause all three types of infection, but M1 is overrepresented in symptomatic infections whereas M12 is more prone to be asymptomatic [11,12]. Similarly, a large study performed at the Centers for Disease Control and Prevention (CDC) found that the M1 serotype was significantly associated with invasive infection and M12 with noninvasive infection [13]. The type of infection is thus determined by both the host and the pathogen.

In this opinion article we focus on the contribution to virulence made by the pathogen and ask: why does GAS have the ability to cause three different types of infection with distinct levels of virulence? To address this question, we integrate mechanistic understanding from infection medicine with recent theory from evolutionary ecology. Both the evolutionary theory of virulence and the study of molecular virulence mechanisms are well developed, but the two fields are rarely combined as an integrated whole. Here we provide such a synthesis for an important human pathogen. We consider GAS as a case in point, but the outlined scenario should be applicable to other pathogens that have a similar virulence pattern and are adapted to their host rather than infect it only accidentally.

GAS and virulence evolution theory

A foundation of contemporary virulence evolution theory is the 'trade-off hypothesis'. One version of this may be summarized as follows. The more the bacteria replicate

and the higher densities they attain in the host, the higher the rate of transmission to new hosts (number of daughter infections per unit time). However, high replication and bacterial load result in virulence, by damaging the host, and limit the time available for transmission; for example, by eliciting a protective immune response that clears the infection [14] or by killing the host [15]. From these assumptions, it is concluded that virulence is associated with both positive and negative effects on bacterial transmission. The optimal strategy for the pathogen is therefore an intermediate level of virulence (see Box 1 and [15] for details).

The assumptions behind the trade-off hypothesis are supported for GAS. First, there is evidence for associations between bacterial load and transmission rate [16] and between transmission and symptomatic infection [17]. Second, virulence (symptoms) correlates with bacterial load [18] and third, the duration of asymptomatic colonization is much longer than that of superficial symptomatic infection [10].

There is, however, a problem. Given that the assumptions of the trade-off hypothesis apply to GAS, the expectation is that GAS evolutionary trajectories should converge on a single optimal level of virulence, but this is not what is observed. GAS cause three distinct types of infection with virulence levels ranging from the entirely innocuous to the highly lethal. This means that the basic trade-off model is unable to account for the virulence pattern seen in GAS.

Box 1. The trade-off hypothesis of virulence evolution

Here we summarize the line of thought associated with the trade-off hypothesis.

The Darwinian fitness of a pathogen is determined by its ability to transmit to new host individuals. For a pathogen to persist in the host population, infections need to produce on average at least one daughter infection, so that $R_0 \geq 1$, where R_0 denotes the number of new infections arising from an infected host in a population where all hosts are susceptible. By contrast, if $R_0 < 1$, the pathogen will eventually become extinct. When there is variation in R_0 among different genotypes of a pathogen, natural selection should favour those with characteristics that result in higher R_0. The question is then, how does virulence relate to R_0 or, in other words, how does natural selection act on virulence?

Most models of virulence evolution have focused on virulence in the sense of infection-induced mortality of the host and assumed that increasing pathogen replication within the host increases virulence. Thus, increased replication comes at a cost for the pathogen because host death reduces the time span available for transmission to other hosts. This led to the 'conventional wisdom' that prevailed until the 1980s, that pathogens should eventually evolve avirulence to maximize their Darwinian fitness [55]. However, increased replication may also benefit the pathogen (i.e., increase R_0) by increasing the rate of transmission to new hosts (i.e., the number of daughter infections per unit time). If replication affects both virulence and transmission rate, fitness (i.e., R_0) may be maximized at some intermediate level of virulence rather than at infinitesimal virulence as under the 'conventional wisdom' scenario. An intermediate optimum for virulence can also come about in several other ways than through a trade-off between infection-induced mortality and transmission rate. For example, if higher pathogen replication induces a stronger immune response, there may be an intermediate optimal level of virulence as a result of a trade-off between transmission rate and immune clearance [14]. Thus, when virulence has not only costs, for example reduced time span for transmission, but also a benefit in the form of increased transmission rate, natural selection can favour an intermediate level of virulence rather than avirulence, an idea that has become known as the trade-off hypothesis. Assumptions of the trade-off hypothesis have now been addressed and confirmed in several host–pathogen systems. The reader is referred to [15] for a review of the trade-off hypothesis and its empirical support. In the main text we discuss some possibilities that go beyond this basic scheme.

Which infection types are actually adaptive?

Explanation by optimality models, such as those based on the trade-off hypothesis, requires that the phenomenon to be explained represent adaptation. There are reasons to believe that asymptomatic and superficial symptomatic GAS infections are adaptive, whereas invasive infections are not. First, the two noninvasive types of infection dominate GAS natural history, whereas invasive infections constitute a minute proportion [1]. Second, both asymptomatic and superficial symptomatic infections are likely to transmit, whereas transmission from the blood stream and deep tissues seems unlikely. If the bacteria do not transmit, the infection type cannot be adaptive. Third, many invasive isolates have mutations in the CovRS regulatory system, but these are most probably a result of *de novo* mutation and evolution within the host individual from which the bacteria were isolated [19,20]. In an animal model of superficial infection, such bacteria had impaired fitness [21]. In other words, these variants represent evolution of virulence through 'short-sighted evolution' [22], not a long-term evolutionary optimum. We hence conclude that the two noninvasive types of infection are likely to represent two different adaptive strategies, whereas invasive infection does not. Accordingly, asymptomatic colonization and superficial symptomatic infection are both within the scope of optimality models, whereas invasive infection falls outside.

This does not solve the problem, however; the question is merely split in two: (i) why are there two adaptive types of infection rather than only one; and (ii) why is there a non-adaptive type of infection? To address these questions, we first describe how the habitat of GAS allows for two different adaptive strategies and suggest how the bacteria can use them and what side effects this may have. We then discuss more complex evolutionary models, to explain why GAS has evolved both adaptive strategies rather than specialized in one of them.

Two adaptive strategies and an unfortunate side effect

Both of the potentially adaptive types of infection (asymptomatic and superficial symptomatic) are localized to the host epithelia, specifically those of the skin and upper respiratory tract. When healthy, these epithelia are poor in glucose [23,24] and, because glucose is an important nutrient for GAS [25], this makes them poor substrates for GAS proliferation. By contrast, when the epithelia are inflamed, the influx of a plasma exudate supplies glucose and other nutrients, which can be exploited by the bacteria [23,26,27]. However, the function of the inflammatory exudate is hardly to feed pathogenic bacteria. Rather, it is part of the host's innate immune defence and besides nutrients it contains a range of factors that are deleterious to microbes [28]. Thus, the epithelium has two states. When healthy, it is nutrient poor; when inflamed, it is

nutrient rich, but also defensive. Because nutrients are required for replication, this means that the epithelium may support two different bacterial strategies. In asymptomatic colonization, nutrients are scarce, bacterial replication rate is low, and host defences are not activated. This results in long infections with a low rate of transmission between hosts. In superficial symptomatic infections, there are more nutrients, the replication rate is higher, and the host's defences are activated. This results in short infections with a high rate of transmission (Figure 1A).

The strategy associated with exploitation of the epithelium's nutrient-rich state requires at least three bacterial traits. The first is the induction of inflammation and is conferred by proinflammatory virulence factors. Examples include the superantigens, which activate large populations of T cells and thereby elicit inflammation [28]. The resulting inflammatory exudate contains both immune components and nutrients. Accordingly, the second trait required is evasion of immunity. This trait corresponds to virulence factors such as antiphagocytic proteins and factors inactivating antimicrobial peptides. The third trait is the ability to exploit the supplied resources, which includes both sequestration and metabolism. The system is summarized in Figure 1B.

An example of a molecular mechanism involved in exploiting the inflamed epithelium is the M protein, a key virulence factor in GAS [3]. In symptomatic infection (pharyngitis) it is highly expressed, whereas in asymptomatic colonization it is downregulated [18,29]. It contributes to all three traits discussed previously as required for the exploitation of inflammation: it has proinflammatory activity [30], it protects against phagocytosis [31], and it is involved in nutrient acquisition [26]. Expression of the M protein is coregulated with numerous immune-evasive and other virulence factors by the multigene activator (Mga) [also known as the virulence regulator (VirR)] [32]. Mga also regulates metabolism [32]. It is associated with the exponential phase of growth [33] and is probably linked to glucose availability and utilization [34]. In a primate model of superficial infection, the transcription of *mga* and several key virulence genes, including *emm* (encoding the M protein) and genes for superantigens, were monitored and found to correlate with symptoms [18]. In summary, expression of the Mga regulator and the M protein virulence factor contributes to the induction, evasion, and exploitation of inflammation and these factors are upregulated in the symptomatic setting and downregulated in the asymptomatic setting.

Although these traits are adaptations for exploiting the epithelium's nutrient-rich state, they may also explain why GAS are capable of producing invasive infections. In a local infection in the pharynx, the induction, evasion, and exploitation of inflammation result in pharyngitis. However, if the bacteria accidentally enter a normally sterile site, the same traits would allow them to grow rapidly, make it hard for the immune system to eradicate them, and induce a systemic immune response. The result would be a systemic inflammatory response syndrome (SIRS) due to infection, which is the definition of sepsis [35]. On a more general note, the plasma, nutrients and host defences present at the inflamed epithelium are similar to those in the invasive situation. Therefore, adaptations for the inflamed epithelium can also exert their effects at normally sterile sites, but the effects for the host are then more severe (Figure 1C). In line with this, the M protein discussed earlier is important in invasive disease [2].

In summary, we have proposed that the epithelium allows for two strategies, corresponding to the adaptive types of infection, and that invasive infection arises as a side effect of adaptations for one of those strategies. We now turn to why GAS would have evolved two adaptive strategies rather than only one.

Why are there two adaptive types of infection?

The trade-off hypothesis, which predicts a single intermediate, optimal level of virulence, is very general and simplified, which is both its strength and its weakness. More recent models consider more factors and yield a more complex picture. In particular, there are now models that allow coexistence of two distinct strategies, where one strategy has low virulence and the other has high. Here we discuss three different but not mutually exclusive scenarios.

Boldin and Kisdi showed that pathogens that transmit both directly between hosts and indirectly via the environment can have two adaptive levels of virulence, where the lower level is optimal under direct transmission and the higher under environmental transmission [36]. GAS transmit directly [3] but are also shed into the environment where they remain viable for months, and shedding of large numbers of bacteria is associated with high nasal bacterial load [37,38]. These fomites do not seem to give rise to pharyngitis, however, at least not when dry, but can result in other infections [39–43].

Another model allowing the coexistence of different levels of virulence was proposed by Brown *et al.* [44]. Here, a pathogenic strain gains a competitive advantage over a commensal by inducing an immune response that the commensal cannot cope with. This strategy requires that the pathogen can both provoke immune responses and protect itself against them, which fits well with the strategies and virulence factors of GAS. The scenario allows the coexistence of pathogenic and commensal strains in the population [44]. The models of Brown *et al.* and of Boldin and Kisdi were not developed specifically for GAS. Nevertheless, they show that, when some complexity is allowed for, a pattern with two adaptive levels of virulence can emerge.

The epidemiology of GAS involves abundant asymptomatic carriage alternating with outbreaks of symptomatic infections. Based on this pattern, we propose a third scenario, where the two adaptive infection types represent adaptation to different epidemiological phases. The trade-off model of virulence evolution reviewed previously is based on the idea that the Darwinian fitness of a pathogen is maximized by maximizing the number of daughter infections to which a primary infection gives rise in a fully susceptible host population (R_0) (Box 1). However, this assumes that the population dynamics of both the pathogen and the host have reached equilibrium, with a stable prevalence of the pathogen and a stable density of susceptible hosts, which means that the pathogen is in endemic

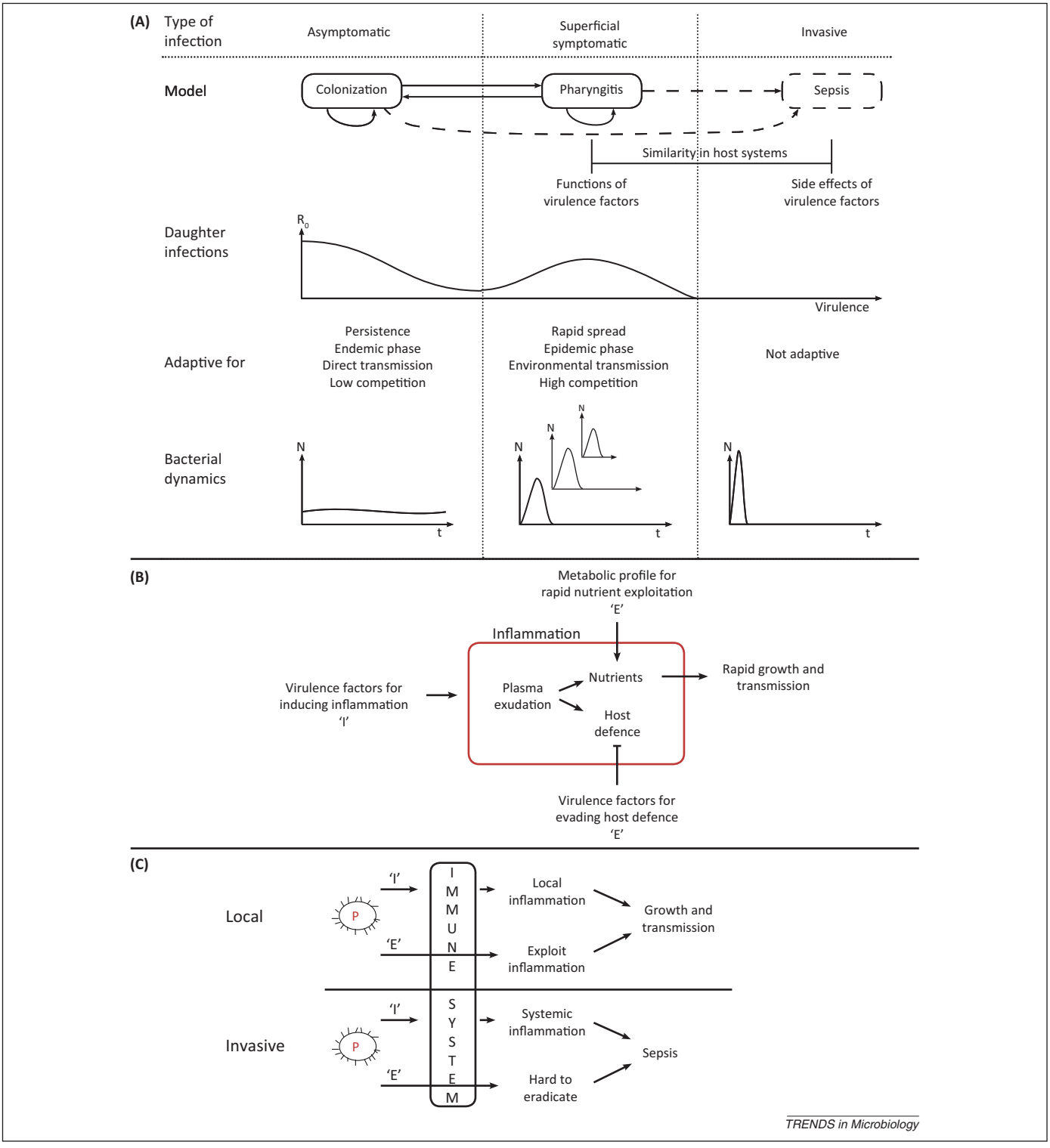


Figure 1. Infection and adaptive strategies of group A streptococci (GAS). **(A)** Types of infection. There are three distinct types of infection (*sensu lato*) with GAS: asymptomatic (colonization), superficial symptomatic (e.g., pharyngitis), and invasive (e.g., sepsis). These are here represented in a model with three compartments. Arrows indicate transmission; that is, infections of one type giving rise to new infections of the same or a different type. Solid arrows represent frequent transmission events and dashed arrows represent infrequent events. Asymptomatic colonization and superficial symptomatic infection are different strategies and involve different kinds of interaction with the host, such that symptomatic infection requires virulence factors. They also result in different bacterial dynamics. Superficial symptomatic infections have burst–crash-like dynamics with short infections of high intensity as the bacteria spread from host to host (represented as separate coordinate systems), whereas colonization has a lower intensity but lasts for a longer time. N, number of bacteria in the infection; t, time. Superficial symptomatic infection and asymptomatic colonization may serve different functions for the bacterium, with colonization contributing more to persistence and symptomatic infection to rapid spread, as in outbreaks, and they may be adaptive under different conditions. Invasive infection is depicted as a dead end without function for the bacterium. Virulence is taken in a broad sense. **(B)** Traits required for exploitation of the epithelium's nutrient-rich state. There are virulence factors that induce immune responses and others that counteract them. In principle, this may be interpreted as representing the bacterium being targeted by host defences and defending itself or as a way to achieve a fine-tuned balance. Here we adopt neither of those perspectives. Instead we propose that some virulence factors [induction of inflammation (I)] induce a general inflammatory response. This response has a range of components, many of which are deleterious to the bacteria whereas others (nutrients) are beneficial. The counteracting factors [evasion of immunity and exploitation of inflammation-associated nutrients (E)] more specifically inhibit various deleterious effects, leaving the beneficial ones to be exploited (E) for growth and

(Figure legend continued on the bottom of the next page.)

phase. By contrast, during the epidemic phase, when the prevalence of the pathogen is still increasing, it is more important to maximize the rate of transmission than R_0. This is because, when the prevalence of the pathogen is increasing, the infection that produces the most daughter infections per unit time, rather than the greatest total number of daughter infections, will have the highest representation in future generations. During the epidemic phase, therefore, natural selection favours high transmission rates and virulence even if it shortens the transmission period and thereby lowers R_0 [45]. Hence, a pathogen for which outbreaks (epidemic phase) alternate with equilibrium conditions (endemic phase) could experience selection for two different strategies. In outbreaks, selection would favour high transmission rates at a cost of transmission time span and R_0. The equilibrium conditions would instead select for high R_0, favouring a longer transmission time span at a cost of transmission rate. Alternation between different epidemiological conditions, such that the bacteria experience repeated cycles with epidemic and endemic phases, could potentially maintain the ability for both strategies and the associated types of infection.

An epigenetic mechanism for virulence bistability

As reviewed in the introductory paragraph, certain GAS serotypes are biased towards causing certain types of infection relative to other serotypes, indicating genetic variation for infection type. However, variation among serotypes explains only part of the variation in infection type [11,12,17], the residual typically being ascribed to variation among host individuals. Here we explore another factor that may contribute: bacterial epigenetic regulation of virulence.

Phenotypic bistability due to epigenetic mechanisms has been described in several bacteria and has been argued to be ubiquitous [46]. A common epigenetic mechanism is a regulatory system with positive feedback loops [46]. Positive feedback loops perpetuate regulatory states by continuing the production of regulators after the initial stimulus is removed [47] and because bacteria have no specialized germline but reproduce by division, the regulators and the corresponding regulatory states are inherited by the daughter cells. Depending on the exact characteristics, such systems may result in occasional switching between alternative heritable phenotypes [46]. A positive feedback loop was recently found to mediate bistability in the expression of a virulence factor in *S. pneumoniae* [48]. A similar phenomenon seems likely in GAS. For example, the Mga regulator (discussed above) induces itself in a positive feedback loop and is inhibited by the RofA regulator, which is also regulated by positive feedback [49]. Thus, GAS may have two transcriptional states, one dominated by Mga, with high expression of M protein and other Mga-induced virulence factors, and the other dominated by RofA, with low expression of these factors. These systems may therefore allow GAS to switch between phenotypes appropriate for asymptomatic colonization and superficial symptomatic infection without genetic changes. The existence, heritability, and reversibility of such alternative phenotypic states are supported by data from several studies on GAS [29,50–53]. [By contrast, the CovRS mutations often found in GAS isolated from invasive infections (discussed above) by virtue of being mutations are not part of the epigenetic phenotypic switch described here.]

If bacteria initiating an infection inherit their transcriptional state from their ancestors in the previous infection, and the expression of virulence factors contributes to the induction of symptoms, primary infections should tend to give rise to daughter infections of their own type, regardless of bacterial genotype. This would be a form of epigenetic inheritance at the level of infection and could contribute to the clustering of symptomatic infections in space and time (as outbreaks).

GAS regulatory systems, including Mga and RofA, respond to environmental cues [49]. It is therefore possible that these regulators increase the probability that the transcriptional states of the bacteria match the conditions favouring the different strategies. This is, however, not required for phenotypic switching to be adaptive, but even random switching can be selected for if conditions vary frequently and unpredictably, a phenomenon known as 'bet hedging' [54].

Concluding remarks and future directions

We have argued that asymptomatic colonization and superficial symptomatic infection represent two different adaptive strategies. The two strategies utilize different host niches: the healthy and the inflamed epithelium, respectively. We also suggested that natural selection has maintained both strategies, rather than favoured one of them, because GAS have two modes of transmission (direct and via the environment), experience different levels of competition when establishing an infection, and/or alternate between epidemic and endemic phases. Thus, the adaptive significance of GAS virulence factors is to induce, evade, and exploit inflammation to maximize Darwinian fitness under certain conditions (environmental transmission, competition, and/or the epidemic phase). These same virulence factors occasionally give rise to invasive disease as a non-adaptive side effect. Infection type is partly determined by bacterial genotype but may also be due to epigenetic effects, which make the phenotype heritable for several generations. In Box 2 we list questions that are important to address in evaluating our proposed scenario.

Understanding the adaptive significance of different types of infection with a given pathogen is crucial to predicting the evolutionary response to interventions. For example, antibiotic treatment of adaptive infections often results in the evolution of resistance in the bacterial

transmission. **(C)** Sepsis as a side effect of the traits described in (B). Plausibly, many or most virulence factors are adaptations for the superficial symptomatic type of infection, with its nutritious plasma exudate and activated host defences, and are selected for in that context. Accordingly, traits I and E allow the bacteria to induce local inflammation, evade its antibacterial effects, and exploit the nutrients for growth and transmission. Because the host components encountered in the invasive setting are similar to those at the inflamed epithelium, those same traits are also functional in the invasive environment, but the systemic nature of invasive infection makes the consequences more severe. This has no function for the bacterium, but is a mere side effect.

Box 2. Outstanding questions

- What is the role of environmental transmission in GAS, especially for infections other than pharyngitis (Boldin and Kisdi's model [36])?
- What are the roles of inter-strain competition and competition with commensal microbiota in the establishment of GAS infection (Brown *et al.*'s model [44])?
- How large do GAS epidemics need to be for selection to favour virulence? Would a kindergarten of up to 20 children suffice or is the population of an entire country necessary (our proposal for the existence of two virulence optima)?
- How do the classical virulence factors described for invasive infection increase the Darwinian fitness of the pathogen in superficial symptomatic infections?

population, whereas treatment of non-adaptive infections should not have this effect. We hope that continued cooperation between evolutionary and medical research will result in formal and empirically tested models that can be used to design treatment and prevention strategies that maximize effect and minimize the evolution of resistance.

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Opinion

PVC bacteria: variation of, but not exception to, the Gram-negative cell plan

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Planctomycetes–Verrucomicrobia–Chlamydiae (PVC) bacteria have features that differentiate them from classical Gram-negative (G–) bacteria. One such feature is their complex endomembrane system. Based on the difference of membrane organization and compartment identity, PVC bacteria were proposed to form an exception to the bacterial G– cell plan. Here I argue that all PVC membranes are derived from G– membranes, and that their organization and the compartments they form are similar to those of G– bacteria. I suggest that PVC membrane organization should be evaluated within a G– framework and as a variation of it.

Peculiar characteristics of PVC bacteria

The bacterial PVC superphylum is an assemblage of bacterial phyla that include the Planctomycetes, the Verrucomicrobia, the Chlamydiae, the Poribacteria, and the Lentisphaerae, together with several other groups. Their grouping is now well supported by various phylogenetic approaches and markers [1–6]. Additional support for their relatedness is provided by the occurrence of characteristics that are rare in bacteria, some of which are more commonly associated with archaea or eukaryotes [7,8]. These include the lack of the division protein FtsZ and the production of sterol [3,9]. These characteristics are present in an irregular pattern among PVC members; in other words, not all species have these properties, suggesting that the last common ancestor of the PVCs already had most of these characteristics that were subsequently lost in some lineages [8]. The homology between these features and their non-bacterial equivalent is unresolved and controversial [10–12]. However, their presence in PVC members has led to various hypotheses about the evolutionary relationship of PVC bacteria to eukaryotes and archaea (Box 1).

One of these features is the presence of a complex endomembrane system. Due to some of its characteristics, it has been proposed that PVC bacteria constitute an exception to the Gram-negative (G–) bacterial cell-plan definition (see Glossary) [13–16]. The bacterial G– cell plan is defined by an outer membrane (OM) separated from the cytoplasmic inner membrane (IM) by the periplasm. Inside the periplasmic volume is located the peptidoglycan, or murein, which helps maintain cell integrity, forming the cell wall. The outer membrane typically contains the lipopolysaccharide (LPS) on its outer leaf. The PVC cell-plan exception claims that the outermost membrane in most PVCs is the cytoplasmic membrane, implying that there is no OM, periplasm, and that the cytoplasm is divided in an inner and an outer cytoplasm. However, the evidence supporting this proposal is more than 10 years old and has been challenged by recent data. Here I focus on the claim that PVC bacteria are an exception to the G– cell plan. I review the historical evidence that led to this proposal, summarize recent data, and propose a new interpretation in which PVC bacteria are a variation of, but not an exception to, the G– bacterial cell-plan definition. I then focus on the implications for bacterial definition, evolutionary hypotheses, and future research directions.

Glossary

Division and cell wall (*dcw*) cluster: a gene cluster containing genes involved in peptidoglycan precursor synthesis and in cell division, including the otherwise ubiquitous *ftsZ* gene. The *dcw* cluster is degraded is most PVCs.
FtsZ: the bacterial division protein. It forms concentric rings at the division site in almost all bacteria. Constriction of the FtsZ rings drives the fission of a dividing cell. FtsZ is almost universal and essential in bacteria. FtsZ is usually encoded in the *dcw* cluster. All planctomycetes and some PVC members have lost the *ftsZ* gene.
Gram-negative (G–) bacteria: G– bacterial cells are defined by the presence of two membranes surrounding their cytoplasm. The inner, or cytoplasmic, membrane is separated from the outer membrane by the periplasmic volume inside which the peptidoglycan, or murein, is located and helps to maintain cell integrity, forming the cell wall. The outer membrane typically contains the LPS on its outer leaf. However, determination of the Gram status of an organism is from staining and is not based on architectural features, and a definition based on membrane identity and numbers is more appropriate [30].
Inner membrane (IM): the cytoplasmic membrane of G– bacteria.
Lipopolysaccharide (LPS): the outermost surface structure of most G– bacteria, consisting of large molecules containing lipid A and polysaccharides joined by a covalent bond. These molecules are anchored in the outer leaf of the outer membrane of G– bacteria. With other compounds, it forms the cell envelope.
Outer membrane (OM): the outermost membrane of G– bacteria.
Peptidoglycan (PG): also known as murein, PG a polymer of sugars and amino acids forming the cell wall outside the cytoplasmic membrane of bacteria.
Periplasm: the space located between IM and OM in G– bacteria.
TonB system: the canonical system for active transport across the OM, where it interacts with a family of β-barrel proteins. The TonB complex spans the periplasmic space to interact with TonB-dependent receptors in the OM.

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Box 1. Evolutionary scenarios between PVC, archaea, and eukaryotes

On the relationship between PVC and eukaryotes and archaea. PVC members display features usually not observed in bacteria and that are most commonly associated with eukaryotes or archaea [7,8]. Various scenarios have been proposed to account for this phenomenon. First, the features observed have been claimed to be analogous, not homologous – in other words, there is no direct evolutionary relationship between them and the observed similarity only reflects convergent evolution [10]. Second, accepting the possible homology between PVC and non-bacterial features, three scenarios of evolutionary relationships between PVCs and eukaryotes/archaea have been proposed to date. (i) The eukaryotes are the result of the fusion between a member of the PVC superphylum, perhaps related to *Gemmata obscuriglobus*, and an unidentified archaea [42]. (ii) The similarities observed are the remnants of a eukaryotic-like universal ancestor, which was followed by multiple losses in most lineages [26,43]. (iii) The observed similarities are illustrations of the transition between an ancestral PVC member and the last archaea and eukaryote common ancestor [7,8].
On the eukaryotic endomembrane system origin. In the 1980s, Blobel and de Duve separately proposed that internalization of the G– periplasm was at the origin of the eukaryotic endomembrane system [40,41]. This proposal was based on the commonalities found between both systems, including protein translocation mechanisms [44], chaperones [45], the unfolded protein response [46], the presence of OM vesicles [47], multidrug resistance efflux pumps [48], and kinases [49].

A bacterial cell-plan exception

The dichotomy between prokaryotes and eukaryotes has been one of the pillars of biological sciences since the discovery that some cells package their DNA in a membrane-defined compartment (eukaryotes) whereas others do not (prokaryotes). Woese's subdivision of the latter in two domains, Archaea and Bacteria, suggested that the dichotomy was no longer appropriate, but did not touch on the criteria of presence or absence of a nucleus [17]. A plethora of cellular, molecular, genomic, and phylogenetic information has confirmed the differences, but also the commonalities, and thus common ancestry, between the three types of cells [18].

The report of a bacterium whose genetic material was surrounded by a double-membrane threatened the prokaryote/eukaryote dichotomy [19]. This exception was the planctomycete bacterium *Gemmata obscuriglobus*. The initial report was followed by others providing more images of the presence of a double-membrane surrounding the DNA in this species [14,15,20]. An early 3D reconstruction based on electron microscopy (EM) provided additional support for the enclosure of the genetic material in a nucleus-like structure [14].

Subsequently, RNA was reported to have been detected in the compartment sandwiched between the outermost and the internal membrane of the planctomycetes *Pirellula marina* and *G. obscuriglobus* [13,14]. This compartment is always devoid of ribosomes. The presence of RNA was interpreted as evidence that this compartment is not equivalent to the G– periplasm but is instead a specialized part of the cytoplasm because RNA was thought to be restricted to the cytoplasm. The interpretation of the cytoplasmic identity of this compartment implied that the outermost membrane was not equivalent to the OM of G– bacteria but constituted the cytoplasmic membrane. This was supported by the appearance of this outermost membrane when observed by EM. In G– bacteria the outer membrane (OM) is asymmetrical because the inner leaf is composed of phospholipids whereas the outer leaf is constituted by the LPS, and this difference is visible by EM. This asymmetry is not observed in the outermost membrane of planctomycetes [13,14,19]. The next internal membrane of planctomycetes was then regarded as being intracytoplasmic membrane.

Thus, it was proposed that the planctomycetal cell plan differs from that of G– bacteria, forming an exception to the bacterial cell-plan definition [15,16]. In this hypothesis, cytoplasmic compartmentalization occurs in planctomycetes, with an outer and an inner cytoplasm, and ensuing differences in membrane identity and organization (Figure 1). This exception to the bacterial cell plan is further complicated in *G. obscuriglobus* with an additional membrane surrounding the genetic material. The absence of peptidoglycan (PG) in planctomycetes supported this exception to the cell-plan hypothesis [21]. Localization on the OM of the F-ATPase in the anaerobic ammonium-oxidizing (anammox) bacteria is also frequently presented as another argument supporting this hypothesis [22]. This exceptional cell plan was recently extended to the Verrucomicrobia and proposed to be already present in the PVC common ancestor [23]. These data were the best available when they was first examined more than a decade ago, and their interpretation was justified. In the subsequent years new data have enriched our knowledge of PVC cell biology and suggested an alternative interpretation.

Revisiting the exception to the bacterial definition

The early observations of a membrane surrounding the genetic material of *G. obscuriglobus* were based on 2D slices only. Even then, more than one 'DNA compartment' was observed in the same cell, incomplete enclosure of the DNA was observed in others, and sometimes a combination of both [14]. These observations already hinted at the fact that the 'nucleus-like' structure might be neither as closed nor as simple as proposed. The early 3D reconstruction was of excellent quality at the time, but it was reconstructed with less than 100 slices of excellent resolution at the time, but that is limited as compared to current tools. I argue that a convincing demonstration of DNA enclosure by a membrane in a bacterium is still missing [24].

The existence of RNA in the periplasm is an interesting possibility with fascinating implications. However, it is unclear if the presence of RNA indicates whether this is a cytoplasmic compartment. To the best of our knowledge, there is little to no evidence that RNA cannot be present in the bacterial periplasm, a compartment that remains poorly characterized. In fact, the existence of periplasmic ribonuclease I (RNase I) supports this possibility [25]. In addition, the statistical significance of periplasmic RNA detection is unclear and few controls were presented. Unfortunately, RNA detection has not been repeated since in this or another PVC member. Thus, these data are to be interpreted cautiously, and additional analysis will be necessary to confirm this possibility. Similarly, the OM localization of the F-ATPase [22] is frequently presented as an argument that the anammox OM is energized and thus

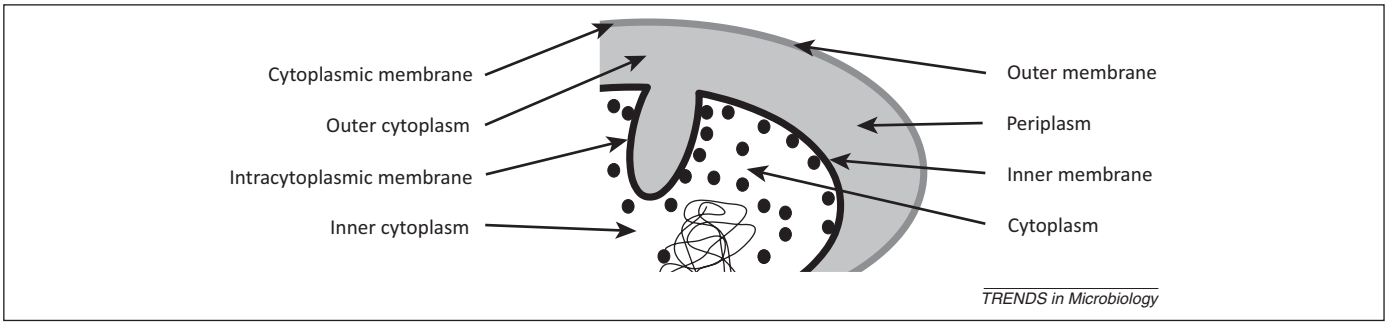


Figure 1. Historical and proposed PVC cell-plan interpretations. Schematic representation of the typical membrane organization of PVC bacteria. Observations of Planctomycetes (middle) were previously interpreted as reflecting cytoplasmic membranes and intracellular compartmentalization of the cytoplasm by the intracytoplasmic membrane (left), whereas the structure is interpreted here as most closely resembling the membrane organization and compartments of G– bacteria (right). DNA is represented as a string. Spheres represent ribosomes. Ribosomes are observed in the inner compartment, never in the outermost one.

corresponds to the cytoplasmic membrane [16,26]. I would like to argue that, in the same publication, F-ATPase was observed at a similar frequency both on the IM and, at a somewhat lower frequency, on the anammoxosome membrane [22]. In conclusion, F-ATPase is observed on all anammox membranes and thus fails to assign any membrane identity.

Overall, the arguments presented for the planctomycetal cell plan being different from that of G– bacteria are unconvincing, and recent genomic and microscopic evidence have prompted the need for their re-evaluation.

Genomic evidence for G– cell plan in PVCs

The major specific constituents of the G– OM are outer membrane proteins (OMPs) and LPS (Box 1). OMPs have a typical β-barrel fold, and proteins with this structural signature were predicted in all Planctomycetes and Verrucomicrobia genomes, and their expression was detected in two Planctomycetes, suggesting the presence of a typical OM in PVCs [27]. LPS is another marker of the G– OM and is anchored in the outer leaf of the OM by lipid A. Genes for the biosynthesis of LPS are present in Planctomycetes genomes and, although unusual, lipid A has been detected in Planctomycetes [28]. These data suggest that Planctomycetes contain at least a partial LPS synthesis pathway [29,30]. OMPs and lipid A also require specific OM insertion mechanisms. Both pathways of lipid A and OMP insertion have been detected in Planctomycetes and Verrucomicrobia genomes [27]. Genes encoding TonB, the canonical system for active transport across the G– OM, including TonB and TonB-dependent receptors, are present in all Verrucomicrobia but were found in only four Planctomycetes genomes. In addition, the division and cell wall synthesis (*dcw)* cluster, including *ftsZ* (involved in cell division) and PG synthesis genes, either complete or as remnants, is present in the genome of all PVC bacteria [3]. The TonB and *dcw* data demonstrate an ancestral presence followed by differential losses, a recurrent theme in PVC genomics. Thus, all PVC members are derived from a G– bacterium. The fact that the *Chlamydiae*, G– bacteria with both an IM and an OM (but which may lack a PG cell wall), belong to the PVC superphylum is now strongly supported and acknowledges their G– ancestry [5,6].

Thus, biochemical, genomic, and expression evidence demonstrate that the PVC ancestor was a G– bacterium with an IM, OM, OMPs, LPS, and a complete *dcw* cluster. Divergent evolution led to characteristics being lost in some species but being retained in others. This applies to the *dcw* cluster whose degradation pattern can be followed in the PVCs; it was lost in Planctomycetes but is still present in Verrucomicrobia and Chlamydiae. In addition, TonB system genes are absent from most Planctomycetes [27], and the LPS has been replaced by a proteinaceous envelope, at least in the Planctomycetes. This suggests that the differential appearance of Planctomycetes and G– OMs is due to the loss of LPS in the former.

EM evidence for a G– cell plan in PVCs

Recent EM analysis has provided compelling evidence against the PVC bacterial exception. Previous cryo-electron tomography implied that the double-membrane network of *G. obscuriglobus* cells emanates from the IM to divide the cytoplasm into unsealed compartments [31]. In agreement, it was already observed that the nucleoid is not entirely surrounded by the double-membrane system.

G. obscuriglobus cell organization has been further described in two cell types [32]. The first has increased periplasmic volume populated by vesicle-like structures. In the second the periplasm is not well developed and no vesicles are observed, but the IM protrudes deeply into the cytoplasm to form thin membrane-sheet invaginations extending towards the inside of the cell. The volume of complete cells of this second type was recently reconstructed at high resolution and the entire organization of internal membranes was followed within the cell [24]. Membrane continuity was reported between the IM and the invaginations in *G. obscuriglobus*, and also the lack of a nucleus-like structure. The volumes created by the membrane invaginations were shown to be interconnected and not closed. These detailed 3D reconstructions revealed that these bacterial cells are neither compartmentalized nor nucleated. There is no nucleus and thus no nuclear pore complex in these bacteria. Thus, the organization of the cellular space in *G. obscuriglobus* is similar to that of a classical G– bacterium, modified by the presence of large invaginations of the IM inside the cytoplasm.

Variation of the membrane organization observed in PVC members suggests that their last common ancestor already possessed the means to manipulate membranes. This appears to be related to the presence in some PVC

proteomes of proteins bearing structural similarities to the membrane coat proteins involved in membrane organization and maintenance in eukaryotes [32]. However, the evolutionary relationship between the bacterial and eukaryotic proteins is still not clear [10,11]. The endomembrane system and membrane coat proteins are linked to the endocytosis-like capability of *G. obscuriglobus*, a unique feature in bacteria [33].

A unified cell plan for PVC bacteria embedded in the classical G– plan

I argue here that an alternative interpretation of the membrane identities and cell plan is more appropriate in view of recent evidence. I propose to interpret PVC membrane organization in a G– bacteria framework – in other words, that there are only two main membranes in most PVC members, and that those membranes are derived from, and are equivalent to, the IM and OM of G– bacteria (Figure 1) [30]. There is no doubt that membrane biochemistry and molecular properties have changed, for example the OM has lost the LPS in most Planctomycetes. However, they are derived from, and are equivalent to, their G– counterparts. I agree that PVCs are exceptional in terms of membrane organization. However, their cell plan is G– derived and all membranes are related to G– membranes. Accordingly, there are only two main volumes in most PVC cells, the cytoplasm and the periplasm, with the possible exception of the anammox (Box 2). Support for this interpretation includes the fact that ribosomes line the various internal membranes in most PVC members, as they do along the IM of G– bacteria. This applies to the central ovoid region membranes in *Isosphaera pallida*, to the invaginations in *G. obscuriglobus*, including the internal face of the nucleus-like structure, the cytoplasmic face of the anammoxosome membrane, and to all other IM-derived membranes in PVCs.

The organization of most membranes in PVC members can be linked in a continuum of variations of periplasmic space volume and IM invaginations or OM evaginations (Figure 2). Classical G– bacteria have a thin periplasmic space sandwiched between the IM and the OM, without particular folding or invaginations of the IM or the OM (Figure 2A). Chlamydiae, members of the PVC superphylum, are G– bacteria with a coccoid or rod shape (Figure 2B). I interpret the situation observed in the planctomycetes *Pirellula marina* and *Pirellula staleyi* as being similar to a G– organization, but with an increased periplasmic volume without IM invaginations that may reflect only an increase in OM surface area (Figure 2C) [13,14]. The situation observed in *I. pallida* is interpreted as being equivalent to a single large invagination of the IM, giving rise to a large ovoid central region where the volume inside the central region is continuous with the periplasm (Figure 2D) [14]. The anammox situation is similar to a complete enclosure, and internalization of this central region by contraction of the rim of the invagination, with possible detachment from the main IM (Figure 2E) [14]. The situation reported in *Planctomyces limnophilus* is interpreted as being similar to that obtained by one or a few major invaginations of the IM towards the inside of the cell (Figure 2F) [34]. I interpret the situation observed in some *G. obscuriglobus* cells as being similar to a conformation with increased periplasmic volume combined with various thinner invaginations of the IM towards the inside of the cell (Figure 2G). It is easy to see how a favorable angle of vision in 2D could be interpreted as the membrane surrounding the DNA (Figure 2H) but 3D consideration rules out this possibility. The other type of *G. obscuriglobus* cells is interpreted as similar to the situation obtained by continued increase of the periplasmic volume and the emergence of vesicles (Figure 2I) [32]. The membrane of origin for these vesicles still needs to be defined, but could be both the IM and the OM. Some vesicles are also observed in the periplasm of *I. pallida* [14]. The situations observed in some Verrucomicrobia, such as *Prosthecobacter dejongeii* and *Verrucomicrobium spinosum*, respectively resemble a G– conformation with evagination(s) of the OM at one cell pole or at various sites (Figure 2J,K) [23]. Membrane organization in the Verrucomicrobia *Chthoniobacter flavus* and in strain Ellin514 does not appear to be very different from the classical G– organization, apart from the presence of electron-dense granules and perhaps a slightly enlarged periplasm (Figure 2L). I propose that all other PVC organizations can be similarly interpreted.

The various membrane morphologies observed in PVC bacteria might represent variations in attempts to accommodate an increased periplasmic space via invagination(s) of the IM towards the inside of the cell, mainly in Planctomycetes, or through OM evagination(s) in Verrucomicrobia. The functional role of these membranes is still unclear. The endomembrane system is involved in the process of protein internalization and degradation in *G. obscuriglobus* [33], and segregates a toxic metabolic intermediate from the cytoplasm in the anammox [35]. It is also possible that some PVC membranes are energized.

G– versus exceptional cell plan

In addition, there are precedents for a G– based interpretation of PVC membrane organization: similar IM extensions or invaginations have been observed in magnetotactic bacteria and photosynthetic bacteria, all of which are G– [36,37]. Thus, the observed membrane organizations are explained best not by an exceptional cell plan but by a

Box 2. Outstanding questions

- How could an energized membrane be reconciled with the presence of OMPs in the anammox genome [35]? Where are the OMPs localized in PVC membranes? Similarly, what is the localization of other canonical bacterial plasma-membrane proteins such as Sec protein translocation system components?
- Is the anammoxosome separated from the IM or attached to it (see Figure 2 in main text)? Could a single attachment site have avoided detection so far? Or does it represent one of the membrane-limited compartments in bacteria [37]?
- What defines a compartment? How closed off must a volume be to be considered compartmented? Is the eukaryotic nucleus, with its thousands of pores, a compartment?
- What defines G– bacteria? Can they be defined by the identity and organization of their membranes and/or membrane proteins [50]? What defines the cytoplasm, periplasm, and cytoplasmic, inner or outer membranes of G– bacteria?
- How do FtsZ(–) PVCs divide? What is the role of the GTPase potentially related to division in the anammox [51]?

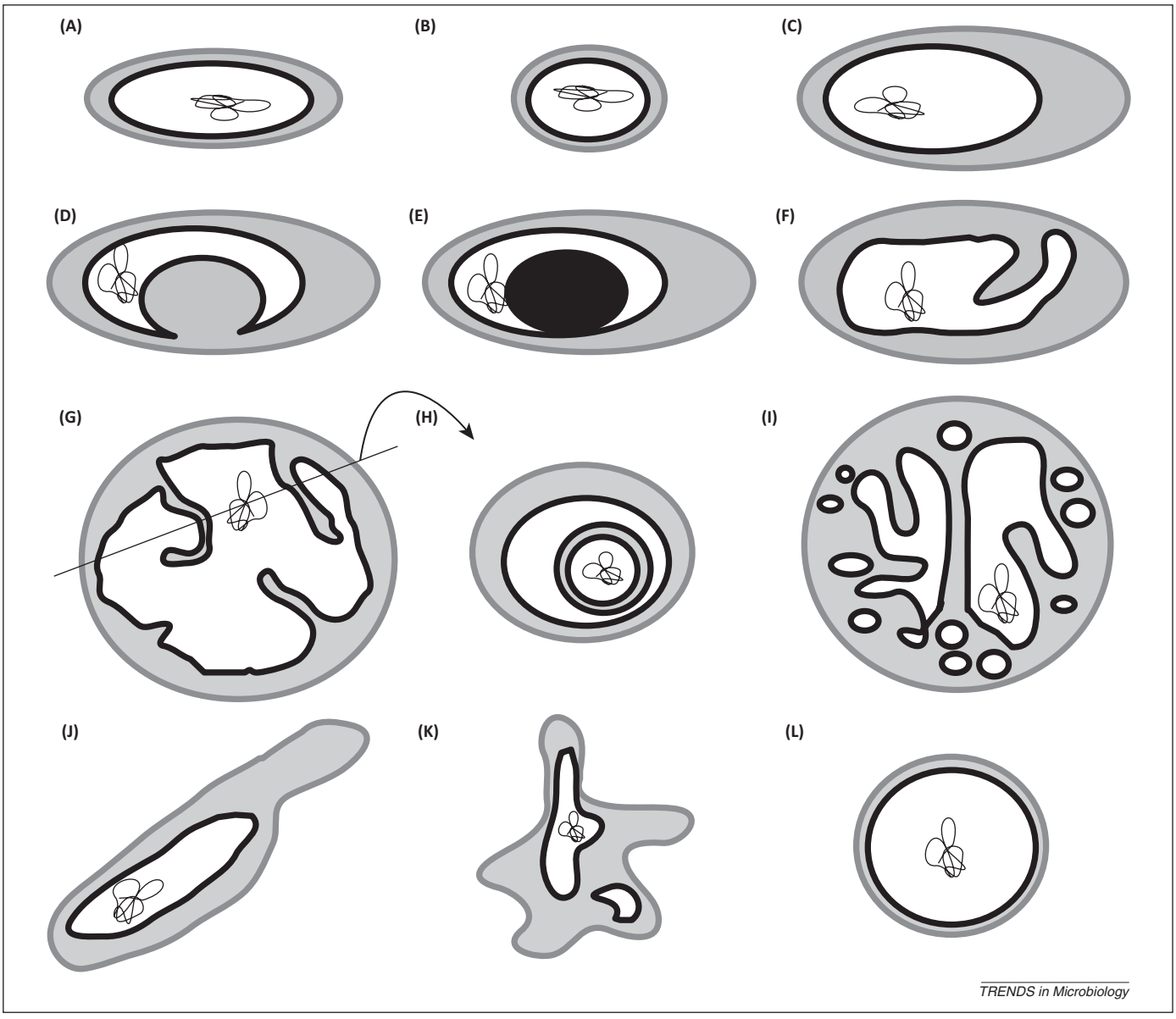


Figure 2. Schema of the cell plan and membrane organization of PVC bacteria. Schematic representation of various cells, with the IM in black and the OM in grey. Cytoplasm and periplasm are in white and grey, respectively. DNA is represented as a string. *Escherichia coli* **(A)** are G– non-PVC bacteria. Chlamydiae **(B)** are G– PVC bacteria. *P. marina* and *P. staleyi* **(C)**, *I. pallida* **(D)**, anammox with the anammoxosome in dark grey **(E)**, *P. limnophilus* **(F)**, *G. obscuriglobus* cell type with invaginations of the IM **(G)**, and *G. obscuriglobus* cell type with vesicles in the periplasm **(I)**. All IM are connected in 3D, even if they don't appear such in 2D. *G. obscuriglobus* might appear to surround its DNA in a compartment when viewed under a favorable angle in 2D only. Arrow points to the representation of the same cell cut along a different angle (line) and observed from the top (**H**). Verrucomicrobia *P. dejongeii* **(J)**, *C. flavus* and strain Ellin514 **(K)**, *V. spinosum* **(L)**. Planctomycetes (C–I) and Verrucomicrobia (J–L) are PVC bacteria. *G. obscuriglobus* (G–I) are Planctomycetes bacteria. Drawings not to scale.

G– derived hypothesis, taking into account evolutionary events known to have taken place in PVCs. Furthermore, exceptional status would imply the lack of an OM. Nevertheless, it is clear that PVCs are G– derived. This is supported by many observations, including the presence of the *dcw* gene cluster (either complete or as remnants) in PVC genomes, that is unlikely to be due to lateral gene transfer [3,27,35,38]. Thus, under the interpretation of exceptional status, the absence of an OM would be more difficult to defend than the presence of an OM in G– bacteria. Exceptional status would also require an explanation of the ribosome-free state of the so-called 'external cytoplasm', in fact the periplasm. This compartment is always observed devoid of ribosomes [13,14]. Explaining this fact in the exceptional status would always be more complex than simply stating that this is the periplasm where ribosomes have never been observed in G– bacteria. If this outermost compartment is part of the cytoplasm, a convincing explanation of how it has become (and how it is kept) ribosome-free has yet to be provided. Again, this is more easily explained in a G– framework.

Evolutionary implications

This interpretation of the PVC cell plan in a G– framework has implications at the genome expression, cell biology, and evolutionary levels.

First, the absence of a membranous compartment completely surrounding the genetic material in *G. obscuriglobus* implies that there is no spatial separation of transcription and translation (at least as a consequence of

complete membrane enclosure). Thus, the prokaryote/eukaryote dichotomy based on this characteristic still stands. This is strongly supported by the presence of ribosomes around the DNA in all *G. obscuriglobus* images.

Second, there is no need to justify the invention of a new cell plan whose origin (between the G– Chlamydiae and the Planctomycetes and Verrucomicrobia phyla) would be difficult to explain. Darwinian evolution could lead from the G– to the PVC cell plan. Bacteria are still defined by two volumes, the cytoplasm and the periplasm, and are not compartmentalized.

Third, this hypothesis bears on the evolutionary link between PVC bacteria and the eukaryotes/archaea (Box 2). Various fusion hypotheses based on G– bacteria have been previously proposed (reviewed in [39]) and the G– variation status of the PVC cell plan could be easily accommodated by most of these. In the case of an eukaryotic-like last universal common ancestor (LUCA), the PVC cell plan might have followed the G– cell plan from which it is derived. However, I believe that the strongest argument for the G– derived PVC cell plan is provided by observations of periplasm internalization [40,41]. The G– derived PVC cell plan supports the hypothesis of periplasm internalization at the origin of the eukaryotic endomembrane system and suggests possible early intermediate steps (Box 1).

Concluding remarks and future prospects

PVC bacteria are still among the most poorly understood organisms, and more than 50% of the genes in their genomes remain of unknown function. Deciphering their molecular and cellular biology is an important goal in the near future. Accordingly, the main players behind most of their particular characteristics are still unknown (which explains why their evolutionary origin is difficult to determine). Isolating and characterizing the molecular actors behind membrane manipulation is one of the next priorities. Membrane coat proteins have been implicated in membrane organization [32], but these proteins are unlikely to work alone and their partners need to be isolated and characterized. The dynamics of the membrane system still needs to be resolved. Localizing the OMPs is another crucial issue because OMPs are markers of the OM and define the identity of the membrane. It will also be important to extend the cell plan and biological mechanisms to other PVC members. In conclusion, even when interpreted in a classical G– framework, a great deal of fascinating biology is to be expected from PVC microorganisms in the future.

Acknowledgments

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Review

Cell targeting by the *Staphylococcus aureus* pore-forming toxins: it's not just about lipids

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Staphylococcus aureus **employs numerous pore-forming cytotoxins to injure host immune cells and promote infection. Until recently, it was unclear how these cytotoxins targeted specific cell types for lysis. Membrane lipids were initially postulated to be cytotoxin receptor candidates. However, the cell-type specificity and species-dependent targeting of these toxins did not support lipids as sole receptors. The recent identification of proteinaceous receptors for several *S. aureus* cytotoxins now provides an explanation for the observed tropism. These findings also have important implications for the implementation of animal models to study *S. aureus* pathogenesis, and for the development of novel therapeutics.**

Bacterial pore forming toxins

The protective lipid bilayer that constitutes the plasma membrane as well as the phagosomal membrane of host cells serves as an interface for host–pathogen interactions, and is commonly targeted by bacterial virulence factors. Such virulence factors include pore-forming toxins (PFTs), which are employed by both Gram-negative and Gram-positive bacteria to cause membrane damage to promote pathogenesis. Although a number of organisms produce PFTs, the bacterial PFTs are the largest and most well studied group of these toxins. Bacterial pathogens utilize PFTs to: cause cell death by membrane disruption as evident with *S. aureus* cytotoxins [1]; translocate toxins or effector molecules across membranes as demonstrated by *Bacillus anthracis* protective antigen (PA) [2]; and/or promote bacterial entry into host cells through the plasma membrane as well as escape from membrane-bound phagosomes as observed with *Listeria monocytogenes* listeriolysin O (LLO) [3,4]. These toxins all exhibit similar properties in that they are typically secreted as water-soluble monomers that interact with membrane components. Once in contact with the target cell membrane, the toxins multimerize into a pre-pore structure that undergoes a conformational change facilitating insertion into the lipid bilayer. Ultimately, a lipid bilayer-spanning pore comprised from each monomer is formed. Although most bacteria produce a limited repertoire of PFTs, a single disease-causing *S. aureus* strain can produce up to six different PFTs that target and kill both innate and adaptive immune cells through osmotic lysis [5].

S. aureus can infect nearly all host tissues and organs, and staphylococcal disease can vary in severity ranging from skin and soft tissue infections to pneumonia, endocarditis, osteomyelitis, and sepsis [6]. The numerous immune evasion tactics utilized by *S. aureus* together with the prevalence of antibiotic resistance make this pathogen particularly difficult to treat. The current methicillin-resistant *S. aureus* (MRSA) epidemic in the community [7], which was previously confined to hospitals, implores a better understanding of *S. aureus* virulence mechanisms and development of alternative treatment strategies.

Until recently, the mechanism by which *S. aureus* PFTs target specific cell types for lysis was unknown, and the only host molecules known to interact with these cytotoxins were membrane lipids. Now, almost a century after the description of the first *S. aureus* cytotoxin, a series of nonredundant proteinaceous receptors have been identified for several PFTs. This review will focus on the identification of these receptors, which has greatly expanded our understanding of cell targeting by the *S. aureus* PFTs and has emphasized the nonredundancy of these PFTs. Importantly, the findings summarized here have broad implications for the development of novel antistaphylococcal therapies aimed at disrupting toxin–receptor interactions.

Cell-targeting by PFTs via specific receptors: from lipids to proteins

Because membrane lipids play an integral role in the pore formation process, lipids and lipid derivatives have long been suspected as receptors for PFTs, and in some cases have proven to be bona fide receptors for PFTs. The cholesterol-dependent cytolysins (CDCs), which form large homo-oligomeric pores comprised of 35–50 monomers, require the membrane lipid cholesterol for pore formation [8]. It is also generally accepted that this large family of toxins, which includes LLO, streptolysin O (SLO) produced by group A streptococci, pneumolysin produced by *Streptococcus pneumoniae*, and perfringolysin O (PLO) produced by *Clostridium perfringens*, utilize cholesterol as a receptor

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[8]. Direct binding to membrane cholesterol has been shown for the prototypical CDC PLO, where three loops located in domain 4 of PLO were found to mediate cholesterol binding [9]. The well-characterized *Aeromonas hydrophila* PFT aerolysin, which is a homolog of the *S. aureus* leukotoxins, does not utilize membrane lipids as cellular determinants but instead shows specificity for the glycosylphosphatidylinositol (GPI) anchors of membrane glycoproteins [10]. It has been proposed that aerolysin recognizes both the GPI anchor and the N-glycan structure of a protein, suggesting that the GPI-linked protein must be properly glycosylated to serve as a receptor for aerolysin [11]. Examples of aerolysin receptors include thymus cell antigen-1 (Thy-1) also known as cluster of differentiation 90 (CD90) [12], contactin [13], and erythrocyte aerolysin receptor [14].

The beginning of this century was a major turning point in the PFT field as it heralded the identification of a proteinaceous receptor for PA, a pore-forming component of anthrax toxin. Although anthrax toxin is actually two binary toxins rather than a PFT, the binding component PA exhibits the properties of a PFT. The heptameric pore formed by PA allows the entry of edema factor (EF) or lethal factor (LF) into target cells [15,16]. Through genetic complementation a splice variant of human tumor endothelium marker 8 (TEM8), which previously had an unknown function, was identified as a receptor for PA and aptly named anthrax toxin receptor (ATR) [17]. Shortly after, capillary morphogenesis protein-2 (CMG2) was identified as a second PA receptor, thus TEM8 and CMG2 are also known as ATR1 and ATR2, respectively [18]. PA directly binds to ATR1 and ATR2 through the extracellular von Willebrand factor type A (VWA) domains of these type 1 membrane proteins [17,18]. The VWA domain is very similar to the ligand binding I domain of integrins [19], thus the β-1 integrin subunit also has been found to serve as a low affinity receptor for PA [20].

Within the past decade, a proteinaceous receptor was also identified for the CDC intermedilysin (ILY) produced by *Streptococcus intermedius*. Although ILY requires cholesterol for pore formation, the preferential targeting of human erythrocytes over non-primate erythrocytes by ILY did not support cholesterol as a receptor for the toxin [21]. Furthermore, treatment of human RBCs with trypsin revealed a proteinaceous influence on ILY binding and hemolysis [22]. CD59, an inhibitor of the pore-forming human complement membrane attack complex [23], was found to mediate binding of ILY to erythrocyte membranes, and was validated as an ILY receptor by gain of function analysis [22]. CD59 is a GPI-linked protein but ILY does not rely on the GPI anchor for receptor binding [22]. The identification of CD59 as an ILY receptor introduced the possibility that not all CDCs utilize cholesterol as cellular receptors. Together with the identification of the ATR receptors, these findings support the possibility that other PFTs, even those with confirmed lipid interactions, may utilize proteinaceous receptors.

S. aureus produces several PFTs with varying cell-type specificities and species-specific tropism

The *S. aureus* cytotoxins, like the CDCs, PA, and aerolysin, are also classified as β-barrel pore-forming toxins because formation of the lipid bilayer-spanning pore occurs through the concerted insertion of the β-hairpin stem domains from each toxin subunit [24,25]. *S. aureus* produces two types of β-barrel PFTs: α-hemolysin and bi-component cytotoxins. α-Hemolysin is the prototypical β-barrel PFT that assembles into a pore through the oligomerization of seven monomeric polypeptides [26,27] (Figure 1). By contrast, the

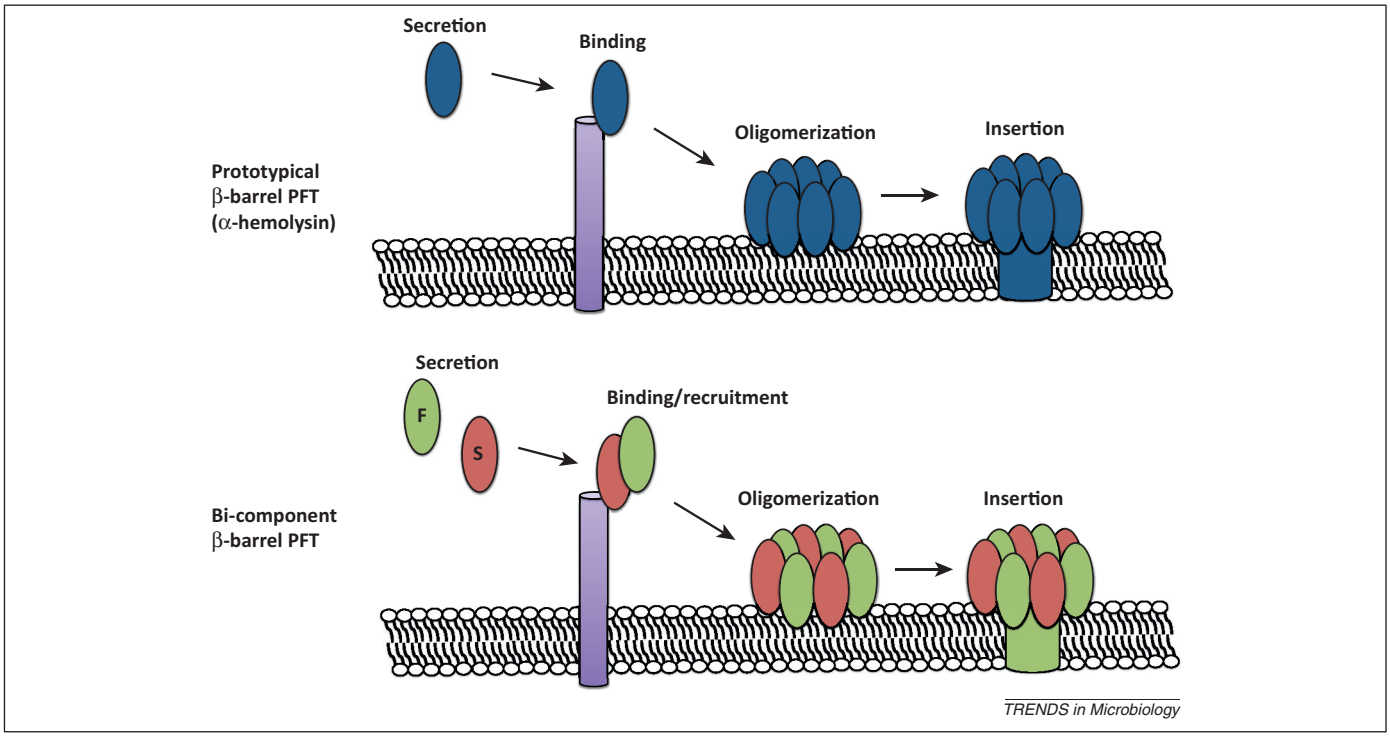


Figure 1. The pore-forming mechanism for the *Staphylococcus aureus* β-barrel pore-forming toxins (PFTs). Diagram showing the proposed pore-forming mechanism for α-hemolysin and the bi-component PFTs. The bi-component PFTs are composed of an S-subunit (S) and an F-subunit (F), where the S-subunit is responsible for receptor binding.

bi-component family of cytotoxins produced by *S. aureus* form hetero-oligomeric pore complexes comprised of two separate polypeptides known as S-class (slow) or F-class (fast) subunits (Figure 1), named for their chromatographic elution profile [28]. A single *S. aureus* strain can produce up to five different bi-component cytotoxins including two forms of γ-hemolysin (HlgAB and HlgCB), leukocidin E/D (LukED), leukocidin S-PV/F-PV (LukSF-PV) also known as Panton-Valentine leukocidin (PVL), and leukocidin A/B (LukAB) also known as LukGH [5]. Ninety nine percent of *S. aureus* clinical isolates have the α-hemolysin (*hla*) and γ-hemolysin (*hlg*) loci [24,29], 87% of clinical isolates contain the *lukED* loci [30,31], we have found the *lukAB* loci in 100% of the clinical isolates evaluated, and 5% of clinical isolates contain the *pvl* genes but 85% of MRSA isolates have *pvl* [32,33]. Additionally, *S. aureus* bovine isolates also have been found to produce leukocidin M/F' (LukMF') [34] and *S. intermedius* produces the bi-component toxin leukocidin S-I/F-I (LukS-IF-I) [35]. Based on structure/function studies with γ-hemolysin and PVL, it has been generally accepted that the bi-component cytotoxins form octameric pores with alternating S- and F-class subunits [25,36–38] (Figure 1). The S-class subunit is primarily responsible for cell targeting, followed by recruitment of the F-class subunit [39] (Figure 1).

Initially, lipids and lipid derivatives appeared to be likely receptor candidates for some of the *S. aureus* cytotoxins. For example, the lipid derivative monoganglioside (G_{M1}) directly binds to the S-class subunits of γ-hemolysin and neutralizes toxin-mediated cytolytic activity [40], suggesting G_{M1} could be a putative receptor for γ-hemolysin. Similarly, the membrane lipid phosphatidylcholine was originally proposed to be an α-hemolysin receptor due to the high-affinity binding observed between α-hemolysin and clustered phosphocholine head groups, and the neutralizing effect of phosphatidylcholine on α-hemolysin binding to and hemolysis of erythrocytes [41]. However, α-hemolysin displays tropism towards rabbit erythrocytes compared to human erythrocytes [42,43], and preferentially targets human lymphocytes and monocytes over neutrophils [44,45]. The species and cell-type specific targeting exhibited by α-hemolysin did not support the sole use of phosphocholine as a receptor. Species and cell-type specificity is also observed for the bi-component leukotoxins. Whereas all the bi-component cytotoxins can lyse neutrophils (also known as polymorphonuclear cells or PMNS) and other leukocytes, γ-hemolysin and LukED also target erythrocytes [30]. Both LukAB and PVL preferentially target human PMNs over rabbit and murine cells, with murine PMNs being the most resistant to the cytolytic activity of these toxins [46,47]. By contrast, LukED does not seem to exhibit species specificity as it has been shown to lyse human, rabbit, and mouse cells [48,49]. The species-specific and cell-type specific targeting of cells, support the hypothesis that specific cellular factors are involved in leukotoxin cell targeting rather than ubiquitous lipids.

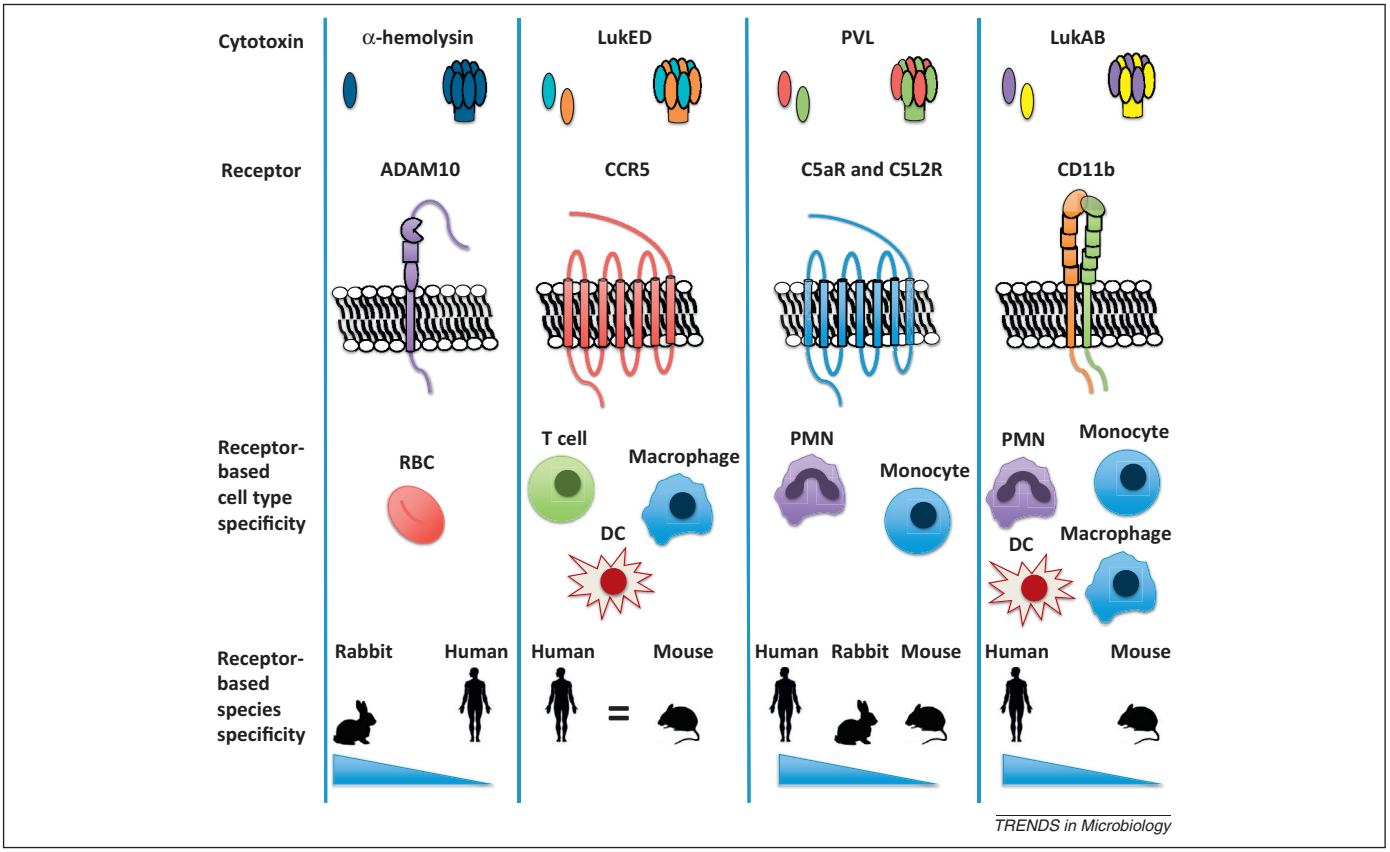


Figure 2. Proteinaceous receptors mediate cell targeting by the *Staphylococcus aureus* cytotoxins. The cytotoxins are listed above, and the number of colors for the different components indicate whether these are prototypical or bi-component pore-forming toxins (PFTs). The results of references [49,50,56,60] are summarized with regard to receptor usage, receptor-mediated hematopoietic cell targets, and receptor-mediated species specificity. Abbreviations: PMN, polymorphonuclear cell or neutrophil; DC, dendritic cell; RBC, red blood cell.

The identification of nonredundant proteinaceous *S. aureus* cytotoxin receptors

Approximately eight decades after the first descriptions of PVL and γ-hemolysin, the first proteinaceous cellular receptor for a staphylococcal PFT was identified. Using a biochemical approach, a disintegrin and metalloprotease 10 (ADAM10) was identified and validated as a cellular determinant for α-hemolysin [50] (Figure 2). ADAM10 is a surface protein that mediates cellular interactions by cleaving membrane proteins with its protease domain [51,52]. The use of ADAM10 as a cellular receptor for α-hemolysin provides an explanation for the species-specific tropism exhibited by this toxin because rabbit, but not human, erythrocytes have ADAM10 on their surface, correlating with the susceptibilities of these cells to this toxin [50] (Figure 2). Whether or not α-hemolysin directly interacts with ADAM10 has yet to be determined, but ADAM10 is required for heptamerization of the toxin [50]. Interestingly, ADAM10-specific binding of α-hemolysin to cells is only observed at low toxin concentrations because higher concentrations of α-hemolysin leads to ADAM10-independent cell binding [50]. This ADAM10-independent cell binding is probably mediated by the interaction between α-hemolysin and phosphocholine head groups.

Multiple proteinaceous receptors were recently identified for several of the bi-component PFTs. The first receptor to be described was for LukED. LukED was found to directly target the seven-transmembrane G-protein coupled chemokine receptor CCR5 [49] (Figure 2). CCR5 is involved in chemotaxis of immune cells and is famously known as a coreceptor for HIV [53–55]. CCR5 targeting by LukED seems to be specific because CCR5 on the cell surface does not render cells susceptible to PVL or LukAB, and additional chemokine receptors such as CCR1, CCR2, CCR3, CXCR4, CCR8, and CXCR6 were ruled out as potential LukED cellular determinants [49]. CCR5 mediates the specific killing of primary human memory lymphocytes, macrophages, and dendritic cells by LukED [49] (Figure 2). Direct binding between the S-class subunit of LukED (LukE) and CCR5 was observed with a dissociation constant (*K*d) of approximately 39.6 nM, as determined by surface plasmon resonance (SPR) [49]. Interestingly, LukED is also cytotoxic towards PMNs and monocytes but in a CCR5-independent manner, indicating the existence of additional LukED receptors [49].

Around the same time that CCR5 was described as the LukED receptor, an antibody neutralization-based approach was used to identify the chemokine receptors C5aR (CD88) and C5L2 (GPR77) as the elusive cellular determinants for the cytotoxic activity of the community-associated-MRSA (CA-MRSA)-linked cytotoxin PVL (Figure 2) [56]. Attempts to block binding of the S-class subunit of PVL to susceptible cells with antibodies targeting different surface receptors revealed that only antibodies against C5aR and C5L2 inhibit the interaction of LukS-PV with cells [56]. C5aR and C5L2 are present on monocytes and PMNs, cells highly susceptible to PVL, but are not found on toxin-resistant lymphocytes [56] (Figure 2). C5aR appears to be the predominant cellular target of PVL based on the high surface levels of C5aR found on PMNs [56]. LukS-PV interacts with C5aR through both the N terminus and the core transmembrane region of C5aR, where the C5aR core is required for pore formation [56]. Importantly, the species specificity exhibited by PVL is explained by the interspecies variation in C5aR. LukS-PV does not interact with murine C5aR but can bind to rabbit C5aR, which correlates with the susceptibility of cells from these species to PVL [56].

Due to the high level of sequence identity between the LukED and PVL toxin subunits, it is perhaps not that surprising that both toxins utilize chemokine receptors to exert their cytolytic activities. γ-Hemolysin is also highly homologous to LukED and PVL because sequence identity among these three toxins ranges from 60 to 80% [57] (Figure 3). Although proteinaceous cellular targets of γ-hemolysin remain to be described, it is likely that γ-hemolysin also uses chemokine receptors, and that overlap may exist between γ-hemolysin and PVL receptors based on toxin homology and binding studies [58].

LukAB is the newest member and most divergent bi-component cytotoxin, exhibiting only 30–40% identity to the other toxins [57,59] (Figure 3). In contrast to the other

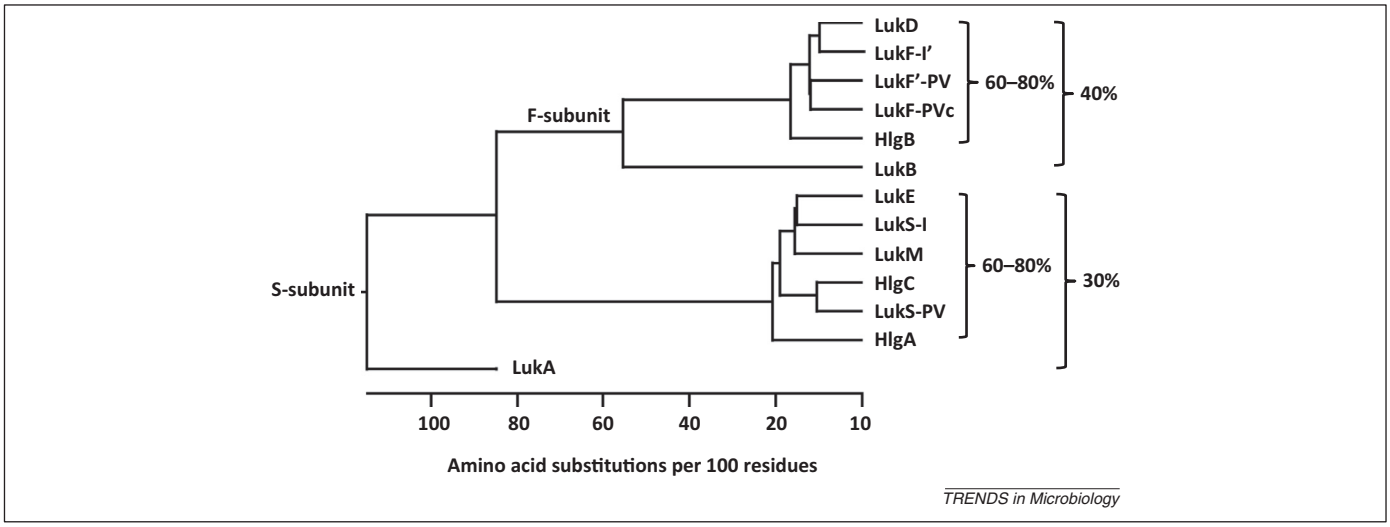


Figure 3. Alignment of *Staphylococcus aureus* cytotoxin subunits. Phylogenetic tree of the mature cytotoxin subunits constructed using the DNASTAR MegAlign Clustal W method. Percentages indicate sequence identities.

bi-component cytotoxins, an integrin subunit rather than a chemokine receptor was recently identified as a LukAB receptor [60]. Through a biochemical approach, the β2 integrin macrophage antigen 1 (Mac-1) (also known as CD11b/CD18, CR3, or integrin αM/β2) was identified as a cellular determinant for LukAB-specific targeting of cells [60]. Mac-1 mediates a number of critical leukocyte functions including phagocytosis, cellular activation, cell-mediated killing, chemotaxis, and adhesion [61,62]. The Mac-1 integrin is highly abundant on the PMN surface as well as the surface of other phagocytes [63] (Figure 2), correlating well with the cellular targets of LukAB [59]. The CD11b subunit of Mac-1 was found to be necessary and sufficient to render cells susceptible to LukAB [60], an effect that is mediated through direct interaction with the I domain of CD11b. The CD11b I domain is required for LukAB cytotoxicity and determines the species specificity exhibited by LukAB [60]. The affinity of LukAB towards the human CD11b I domain is approximately 1.92 nM as determined by SPR [60]. By contrast, no interaction between LukAB and the murine CD11b I domain was observed [60], providing an explanation for why murine cells are highly resistant to LukAB (Figure 2).

The role of cellular receptors in *S. aureus* pathogenesis

The staphylococcal PFTs play key roles in *S. aureus* virulence in *ex vivo* and *in vivo* infection models. Some of these models have been used to explore the importance of cytotoxin–receptor interactions in staphylococcal infections. For example, the murine models developed to study α-hemolysin *in vivo* have proven to be highly informative for elucidating this toxin's contribution to infection, revealing that α-hemolysin contributes to pneumonia, sepsis, and dermonecrosis in mice [64,65]. Moreover, the role of ADAM10 in α-hemolysin-mediated effects in the lungs, skin, and a murine sepsis model has recently been investigated [66–68]. Mice with ADAM10 conditionally knocked out in the respiratory epithelium or the skin display reduced inflammation and better disease outcome [66,68]. *In vivo*, disruption of epithelial barriers seems to be the major outcome of α-hemolysin–ADAM10 interaction because α-hemolysin treatment increases the metalloprotease activity of ADAM10 resulting in specific cleavage of cadherin family proteins in epithelial tight junctions [66–68]. Of note, an oligomerization-deficient mutant of α-hemolysin was not able to increase ADAM10 metalloprotease activity making it difficult to uncouple α-hemolysin pore-mediated cytotoxic effects from ADAM10-mediated effects [66–68].

In contrast to α-hemolysin, the role of PVL in staphylococcal pathogenesis is highly controversial. PVL is epidemiologically linked to strains responsible for CA-MRSA infections such as necrotizing pneumonia and necrotizing skin and soft tissue infections [33,69]. However, elucidating the role of this toxin *in vivo* has proven difficult due to the species-specific nature of the toxin, and has resulted in a number of conflicting studies [64,70–73]. The high resistance of murine PMNs to PVL activity has made studies using murine models of infection difficult to interpret [46]. Despite the resistance of murine cells to PVL toxicity, PVL-mediated inflammatory effects have been observed in the murine lung, which appear to be mediated by Toll-like receptor 2 (TLR2) and CD14 [57,74]. However, binding of PVL to cells transfected with TLR2 and CD14 was not observed by flow cytometry [56]. The identification of C5aR as the PVL receptor, however, suggest that this protein may serve as more than just a docking site for pore-induced killing of cells by PVL because sublytic concentrations of PVL induce C5aR-dependent oxidative burst in human PMNs [56]. Whether or not C5aR-induced priming of PMNs occurs *in vivo* has yet to be determined, but will be difficult to test in murine infection models due to the lack of interaction between PVL and murine C5aR [56].

The identification of C5a receptors [56] and CD11b [60] as cellular targets of PVL and LukAB, respectively, explains the species-specificity exhibited by these toxins, but also exposes the limitations of murine infection models for studying *S. aureus* pathogenesis. The current murine models used to study *S. aureus* pathogenesis seem best suited to study the effect of LukED *in vivo* because the toxin does not appear to exhibit species specificity [48,49]. Infection of human lymphocyte cell lines with CCR5 knocked down, as well as *ex vivo* infection of murine macrophages from CCR5 knockout mice, demonstrate that *S. aureus* kills cells from both species in a CCR5- and LukED-dependent manner [49]. Moreover, CCR5 knockout mice also survived systemic infection with *S. aureus* compared to wild type mice and had a reduced inflammatory response [49]. Similar to PVL, the role of LukAB in murine models of infection is not completely understood. Although LukAB does contribute to bacterial burden in the kidneys of mice during systemic infection with *S. aureus* [59], it is unclear how LukAB promotes bacterial growth in the kidney because murine PMNs are highly resistant to LukAB-mediated killing [47,60]. Because human PMNs are highly susceptible to LukAB, *ex vivo* infection of human PMNs have proved to be more successful at elucidating the potential role of LukAB during infection [59,60,75,76]. From these studies, it is clear that LukAB contributes to *S. aureus*-mediated killing of human PMNs by both extracellular and phagocytosed *S. aureus* by targeting CD11b [60,75]. When employed by phagocytosed *S. aureus*, LukAB facilitates the escape of *S. aureus* from PMNs promoting bacterial growth [60,75].

Currently, the best model to study PVL *in vivo* is the rabbit pneumonia model of infection [77]. This model may also prove useful for studying LukAB *in vivo* due to the fact that rabbit PMNs are more susceptible to LukAB than murine PMNs [47], and because the rabbit CD11b I domain is more similar to that of the human CD11b I domain [60]. In fact, LukAB has been shown to induce inflammation in a rabbit skin model [47], but it is unclear whether this observation is CD11b-mediated. Rabbit PMNs are still 50-fold more resistant to LukAB than human PMNs [47], making the rabbit infection models suboptimal for understanding the role of LukAB in *S. aureus* infections in humans. With the identification of receptors for both PVL and LukAB, generation of transgenic mice engineered to be susceptible to these toxins is now possible, and would be invaluable for studying the receptor-mediated effects of these toxins *in vivo*.

Box 1. Outstanding questions

- Do *Staphylococcus aureus* cytotoxin–receptor interactions promote infection *in vivo*, and if so, does it occur through cytolytic effects or signaling effects, or both?
- As more cytotoxin receptors are identified, will there be overlap among receptors, and if so, are particular cytotoxins employed under certain circumstances *in vivo*?
- With the identification of multiple cytotoxin receptors, can we build an animal model to better mimic human *S. aureus* infection where all *S. aureus* cytotoxins can be studied simultaneously?
- Can cytotoxin–receptor interactions be blocked *in vivo* to inhibit the progression of staphylococcal disease?

Concluding remarks and future perspectives

Previously, the overlap in cellular targets exhibited by the staphylococcal bi-component PFTs has long suggested that these cytotoxins are redundant. The identification of specific, non-overlapping cellular receptors highlight the non-redundant nature of this family of toxins. It is now clear that these PFTs more likely serve as fail-safe for *S. aureus*, ensuring that leukocytes are targeted through independent mechanisms. Moreover, because *S. aureus* targets cellular receptors essential for immune cell functions, the cytotoxins have the potential to cause both cytolytic effects as well as other specific cellular effects through their unique cellular targets (Box 1).

In light of failing antibiotic treatment, the identification of cellular factors that mediate *S. aureus* cytotoxin activity uncovers novel therapeutic targets for the development of treatment alternatives. In fact, the use of CCR5 antagonists currently used to treat HIV infection have already proven successful at blocking LukED-mediated killing of CCR5 positive cells *ex vivo* [49]. Specifically, the CCR5 antagonist Maraviroc inhibits LukED pore formation by blocking the interaction of LukE with CCR5 [49]. These results indicate that disrupting cytotoxin–receptor interactions may be a promising treatment alternative for combating *S. aureus* infections and can possibly be extended to the other cytotoxin–receptor combinations. Overall, the identification of *S. aureus* cytotoxin receptors has provided the necessary tools to better understand how these cytotoxins contribute to *S. aureus* pathogenesis.

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A.L.D. and V.J.T. are listed as inventors on patent applications filed by New York University School of Medicine, which are currently under commercial license.

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Review

The role of bacterial phytotoxins in inhibiting the eukaryotic proteasome

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The ubiquitin-26S proteasome degradation system (UPS) plays a pivotal role in almost all aspects of plant life, including defending against pathogens. Although the proteasome is important for plant immunity, it has been found to be also exploited by pathogens using effectors to increase their virulence. Recent work on the XopJ effector and syringolin A/syrbactins has highlighted host proteasome inhibition as a virulence strategy of pathogens. This review will focus on these recent developments.

The ubiquitin-26S proteasome degradation system (UPS) and plant immunity

Besides the lysosome, the UPS is the main protein degradation system of eukaryotic cells that not only destructs misfolded proteins but is also involved in many cellular processes by degrading proteins regulating such processes [1,2]. Proteins destined for destruction by the proteasome become polyubiquitinated by a reaction cascade involving three enzymes designated E1, E2, and E3. Ubiquitin is activated in an ATP-dependent manner by E1 and transferred to E2, from which it is normally directly transferred to a lysine residue of the target protein by E3, which, by selecting target proteins, confers specificity to the UPS [3]. Polyubiquitination is achieved by multiple rounds in which further ubiquitin units are conjugated to an internal lysine residue of ubiquitin. Polyubiquitinated proteins are substrates for the proteasome, which is composed of two 19S regulatory particles (RPs) capping a 20S core particle (CP). The RPs recognize polyubiquitinated proteins, which become deubiquitinated and unfolded in an ATP-dependent reaction, and regulate access of unfolded substrates to the proteolytic channel of the CP. The channel is formed by four stacked seven-membered rings. The two identical outer rings consist of seven different α subunits, whereas the two identical inner rings consist of seven different β subunits, three of which have proteolytic activities: the β1 subunit exhibits a caspase-like (CL) activity, cleaving protein substrates after acidic residues, whereas the β2 and β5 subunits have trypsin-like (TL) and chymotrypsin-like (ChTL) activities cleaving after basic and hydrophobic residues, respectively. In all three catalytic subunits, the active site residue is the N-terminal threonine (Thr) [4].

The UPS is involved in the signaling pathways of nearly all plant hormones, including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), hormones of particular importance for plant defense against pathogens [5]. SA plays a prominent role in defense reactions directed mainly against biotrophic pathogens [6]. Many of the SA-dependent defense reactions, such as *PR* (*PATHOGENESIS-RELATED*) gene activation, require the key regulator NPR1 (NONEXPRESSOR OF PR GENES 1), which must be turned over by the UPS to function properly [7]. By contrast, JA and ET orchestrate defense reactions mainly directed against necrotrophic pathogens [6]. The JA receptor is part of an E3 ligase targeting JAZ (JA ZIM domain) proteins for proteasomal destruction. The destruction of JAZ proteins releases transcription factors such as MYC2 from sequestration by JAZ proteins and thus leads to the activation of JA response genes [8]. The UPS is also required at multiple steps in ET signaling, such as the regulation of ET biosynthesis and downstream signaling components. In addition to these three well-known defense hormones, other hormones such as auxin, gibberellic acid, and abscisic acid, which all require the UPS in their signal transduction pathways, have also been shown to affect the interactions of plants with pathogens [9]. Apart from hormone signaling, a considerable number of E3 ligases and other UPS components have been found to play a role in plant immunity, further testifying to the importance of the UPS in pathogen defense [10].

Exploitation of the UPS by pathogens

Not only is the UPS involved in many aspects of plant immunity but also a variety of plant and animal pathogens subvert the host UPS for their own good [11]. To see how, it may be helpful to shortly summarize the major paradigms of plant defense against pathogens. As worked out mainly in the model plant *Arabidopsis thaliana*, plants have evolved a two-branched innate immune system to combat pathogens [12]. One branch is named pattern-triggered immunity (PTI) and involves a plethora of physiological and cellular defense responses adverse to pathogens, conferring basal resistance. PTI is launched after recognition of pathogens (or microbe)-associated molecular patterns (PAMPs or MAMPs) by membrane-bound pattern recognition receptors (PRRs), which exhibit structural similarity to the Toll-like innate immune receptors in mammals and insects [13,14]. Bacterial pathogens are able to suppress

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PTI by using their type III secretion system (T3SS) to inject effector proteins (T3Es) into the cytoplasm of host plant cells where they block PTI-associated signaling at many points, thus resulting in effector-triggered susceptibility (ETS) [15,16]. However, plants have evolved a second branch of innate immunity dubbed effector-triggered immunity (ETI), in which specific effectors are recognized by cognate resistance (R) proteins. This usually results in a hypersensitive reaction (HR), a form of local programmed cell death conferring nearly complete but race-specific resistance [17]. In many plants, local HR induces systemic acquired resistance against a broad spectrum of pathogens in remote tissues [18]. T3Es are known to interfere with all these defense reactions at various points [15,16].

Analyses of several plant–pathogen interaction systems have identified a number of T3Es that require the host proteasome for their defense-suppressing activities, thus subverting it for their own use [11]. A well-studied example is HopAB2 (alias AvrPtoB) produced by the tomato pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and some other strains that exhibit E3 ligase activity and target host defense proteins such as PRRs for proteasomal destruction [19–23]. Recently, XopL, a T3E from the pepper pathogen *Xanthomonas campestris* pv. *vesicatoria* suppressing cell death and PTI, was also shown to have E3 ligase activity and to exhibit a novel architecture [24].

Another example is HopM1 produced by *Pst* DC3000 ($HopM1_{DC3000}$), which mediates the proteasomal degradation of AtMIN7 (*A. thaliana* HopM1 Interactor 7), a guanine nucleotide exchange factor required for proper callose deposition, a PTI response [25]. At present, it is not clear whether $HopM1_{DC3000}$ is an E3 ligase itself or whether it recruits an unknown E3 ligase [25]. Furthermore, a T3E family of plant-type F-box proteins (named GALAs) is encoded in the genome of *Ralstonia solanacearum* strains [26], whose members are thought to be part of SCF-type E3 ligases targeting as yet unknown host proteins for destruction [27,28]. GALA7 is necessary for colonization of *Medicago truncatula*, and deletion of all seven *GALA* genes of *R. solanacearum* strain GMI1000 led to loss of pathogenicity on *A. thaliana* and reduced virulence on tomato [28]. In addition, F-box domain-containing proteins have been identified in *Mesorhizobium loti*, *Xanthomonas campestris* pv. *vesicatoria*, and *P. syringae* pv. *phaseolicola*. T3Es exhibiting E3 ligase activity have also been identified in bacteria pathogenic to animals [11], such as the NleG and NleL proteins from enterohemorrhagic *Escherichia coli* [29,30], the IpaH proteins of *Shigella flexneri* [31,32], as well as SspH2 and SopA of *Salmonella* [33,34]. Exploitation of the host proteasome for the enhancement of virulence may be fairly widespread not only in pathogenic bacteria but also in other pathogens such as the oomycete *Phytophthora infestans*, which produces the RXLR motif-containing effector AVR3. AVR3 is translocated into the cytoplasm of host cells where it stabilizes the E3 ligase CMPG1, thereby suppressing CMPG1-dependent cell death triggered by pathogen recognition [35,36].

The examples briefly discussed above show that exploitation of the host proteasome function is employed by a wide variety of pathogens to enhance their virulence. By contrast, inhibition of the host proteasome has only recently been recognized as a virulence strategy of certain pathogens. The rest of the review focuses on this recent development and covers important new research, extending a recent review with a similar topic [37].

Proteasome inhibition as a virulence strategy of plant pathogens

A T3E with proteasome inhibiting activity

An intriguing case of proteasome inhibition to enhance virulence has recently been reported by Üstün and colleagues [38], who identified the first T3E effector that suppresses proteasome activity. The *X. campestris* T3E XopJ was shown to interact with the RPT6 (regulatory particle ATPase 6) subunit of the 19S RP in yeast and *in planta* and to inhibit the proteasome. XopJ belongs to the YopJ T3E family of cysteine proteases/acyltransferases that is widely distributed in plant and animal pathogens as well as in plant symbionts and exhibits a characteristic catalytic triad [39]. Mutation of the catalytic triad as well as of the N-terminal myristoylation motif in XopJ abolished its proteasome-inhibiting ability, suggesting that enzymatic activity and membrane anchoring are required for proteasome inhibition. Comparisons of wild type and mutant strains revealed that XopJ delayed host tissue degeneration and suppressed the accumulation of normal levels of SA and SA-dependent defense responses. It is presently not clear how this works mechanistically in view of the necessary localization of XopJ at the membrane. Future research will without doubt elucidate whether proteasome inhibition is the mode of action also of other members of the YopJ superfamily of T3Es. The study of Üstün and coworkers will probably also stimulate the search for T3E-type proteasome inhibitors outside the YopJ family of proteins in other pathogens.

Syringolin A, a small-molecule proteasome inhibitor and virulence factor

Many potent small-molecule proteasome inhibitors belonging to different structural classes have been isolated from microbes and other organisms such as sponges and bryozoans (in which they probably are produced by symbiotic bacteria). Although they draw intense attention as potential drug leads in the fight against certain cancer types [40], the function of these compounds for the biology of their producers remains in most cases completely unexplored. An exception is syringolin A (SylA), which is discussed below.

SylA was found to be secreted by strains of *P. syringae* pv. *syringae* (*Pss*). SylA is a tripeptide derivative consisting of an N-terminal valine and the two non-proteinogenic amino acids 3,4-dehydrolysine and 5-methyl-4-amino-2-hexenoic acid, which form a 12-membered macrolactam ring. The N-terminal valine is joined head-to-head to another valine residue via an unusual ureido group (Figure 1). SylA is the major variant of a family of related compounds in which the valine residues can be substituted by isoleucine and/or the 3,4-dehydrolysine may be replaced by lysine [37,41–43]. SylA was shown to covalently attach to the catalytic N-terminal Thr residue of all catalytic subunits of the proteasome by a Michael-type 1,4-addition to the double bond at C4 in the 12-membered macrolactam

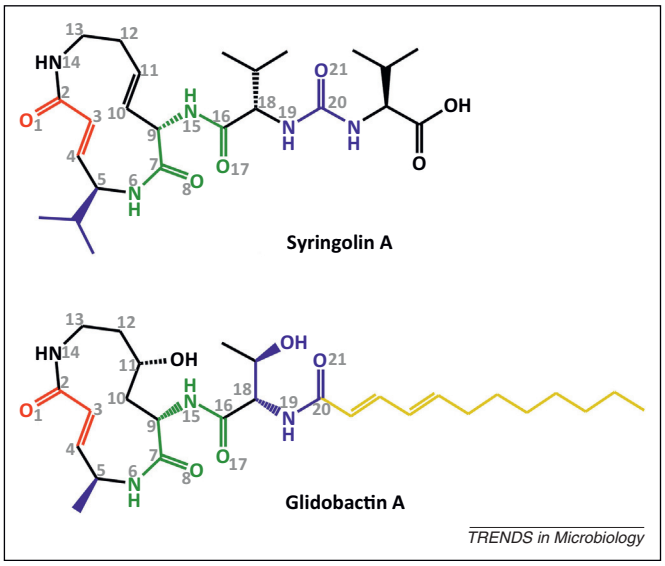


Figure 1. Structure of syringolin A and glidobactin A. The α,β-unsaturated carbonyl group reacting with the N-terminal threonine (Thr) of the catalytic proteasome subunits is given in red. As revealed by crystallography of the inhibitor bound to the yeast 20S core particle (CP), the double bond is opened and the C4 atom becomes linked to the oxygen in the side chain of the N-terminal Thr of the catalytic proteasome subunits, thus forming a stable ether linkage. The dipeptide bond given in green stabilizes the inhibitor upon proteasome binding, whereas the parts in blue determine active site specificity. The aliphatic tail of glidobactin A (GlbA) is shown in yellow. Adapted, with permission, from Macmillan Publishers Ltd (*Nature* [44]), copyright 2008.

ring, thereby irreversibly inhibiting catalytic activity [44]. Proteasome inhibition is probably specific because cysteine proteases and trypsin were not inhibited. Although all three catalytic activities of the proteasome are inhibited *in vitro*, the β5 subunit with its ChTL activity is most sensitive, whereas the β1 subunit conferring CL activity is least sensitive to inhibition by SylA [44]. Similar results were obtained in living plant cells by proteasome activity imaging and profiling experiments, in which SylA seemed to preferentially target proteasomes in the nucleus [45]. As recently elucidated in *A. thaliana*, SylA is efficiently taken up into plant cells by YELLOW STRIPE LIKE7 (YSL7) and YSL8 transporters [46], which belong to a plant-specific oligopeptide transporter subfamily thought to be involved in transport and distribution of metal–nicotianamine complexes [47]. SylA is the founding member of a new structural class of proteasome inhibitors dubbed syrbactins, which is characterized by the 12-membered macrolactam ring and its functional α,β-unsaturated carbonyl group [48,49].

Spray inoculation experiments with the SylA-producing wild type strain *Pss* B728a and a SylA-negative mutant on their bean (*Phaseolus vulgaris*) host revealed the mutant to cause only approximately 30% of brown spot disease symptoms as compared with the wild type, identifying SylA as a virulence factor [44]. Further experiments revealed that SylA not only counteracted stomatal immunity (i.e., the pathogen-induced closure of the stomata, a PTI response dependent on the SA defense pathway and its key regulator NPR1 [50–52]) in bean and *Arabidopsis* but also inhibited other SA-dependent defense reactions such as *PR* gene induction [52]. Inhibition of the SA defense pathway by SylA is most probably due to inhibition of the proteasome-mediated turnover of the key regulator NPR1, which is necessary for its proper functioning as mentioned above. Furthermore, wound inoculation experiments with SylA-producing *Pss* strains on *Nicotiana benthamiana* recently revealed that SylA-mediated suppression of the SA defense pathway also helps the bacteria to escape local wound sites and to colonize adjacent regions along the vascular tissue because acquired resistance was impaired [53].

Occurrence of SylA synthetase genes in *P. syringae* strains

The SylA synthetase, a modular mixed non-ribosomal peptide synthetase/polyketide synthase (NRPS/PKS; see Box 1) is encoded by a gene cluster containing the five genes *sylA*, *sylB*, *sylC*, *sylD*, and *sylE*, the last three forming an operon [54,55] (Figure 2A). Sequence and architecture of these genes implied a SylA biosynthesis model also experimentally supported in most of its aspects [54–58] (Figure 2B). Importantly, the structure of SylA with its 12-membered macrolactam ring containing the functional α,β-unsaturated carbonyl group is reflected in the unique architecture of the *sylD* gene. This allows the identification in genome databases of homologous genes probably encoding structurally similar proteasome inhibitors in other taxa.

A recent genomics analysis of 19 diverse strains belonging to the *P. syringae* species complex revealed complete

Box 1. Non-ribosomal peptide and polyketide synthesis by the thiotemplate mechanism

NRPSs are large modular enzymes which synthesize peptides without the involvement of ribosomes and mRNA templates [70–72]. Each module typically contains condensation (C), adenylation (A), thiolation (T), and optional tailoring domains. Exceptions are starter modules, which normally do not contain a C domain. The A domain catalyzes the adenylation of a cognate amino acid, which becomes attached via a thioester linkage to the phosphopantethein prosthetic group of the T domain. Peptide bond formation between amino acids bound to two adjacent modules as well as release of the upstream amino acid from its T domain is catalyzed by the C domain of the downstream module. Release of the assembled product, which is often cyclized, is catalyzed by the thioeserase (TE) domain in the last module. In linear NRPS, this so-called thiotemplate mechanism leads to an assembly line in which the sequence of the modules as encoded in the genome determines the amino acid sequence of the peptide product (e.g., as in the SylA synthetase). In nonlinear NRPS, modules may act in a sequence different from the one in which they are encoded on the chromosome (e.g., as in the GlbA synthetase).

Modules of type I (modular) PKS usually contain acyltransferase (AT), ketosynthase (KS), and T domains as well as optional ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains. Attachment of coenzyme A-activated malonyl (sometimes methylmalonyl, or, in starter modules, acetyl) moieties to the T domain via a thioester bond is catalyzed by the AT domain. Condensation/decarboxylation of adjacent malonyl residues by the KS domain (which is absent in starter modules) results in a polyketide [70,73]. β-Keto groups can be reduced by the consecutive action of optional KR, DH, and ER domains, resulting in β-hydroxyl groups, α,β-unsaturated double bonds (as in SylA and GlbA), or α,β-saturated bonds, respectively. As in NRPS, the final PKS product is released, often in a cyclized form, by the TE domain of the last module. Similar to the SylA and GlbA synthetases, NRPS and PKS modules often occur together and form mixed NRPS/PKS.

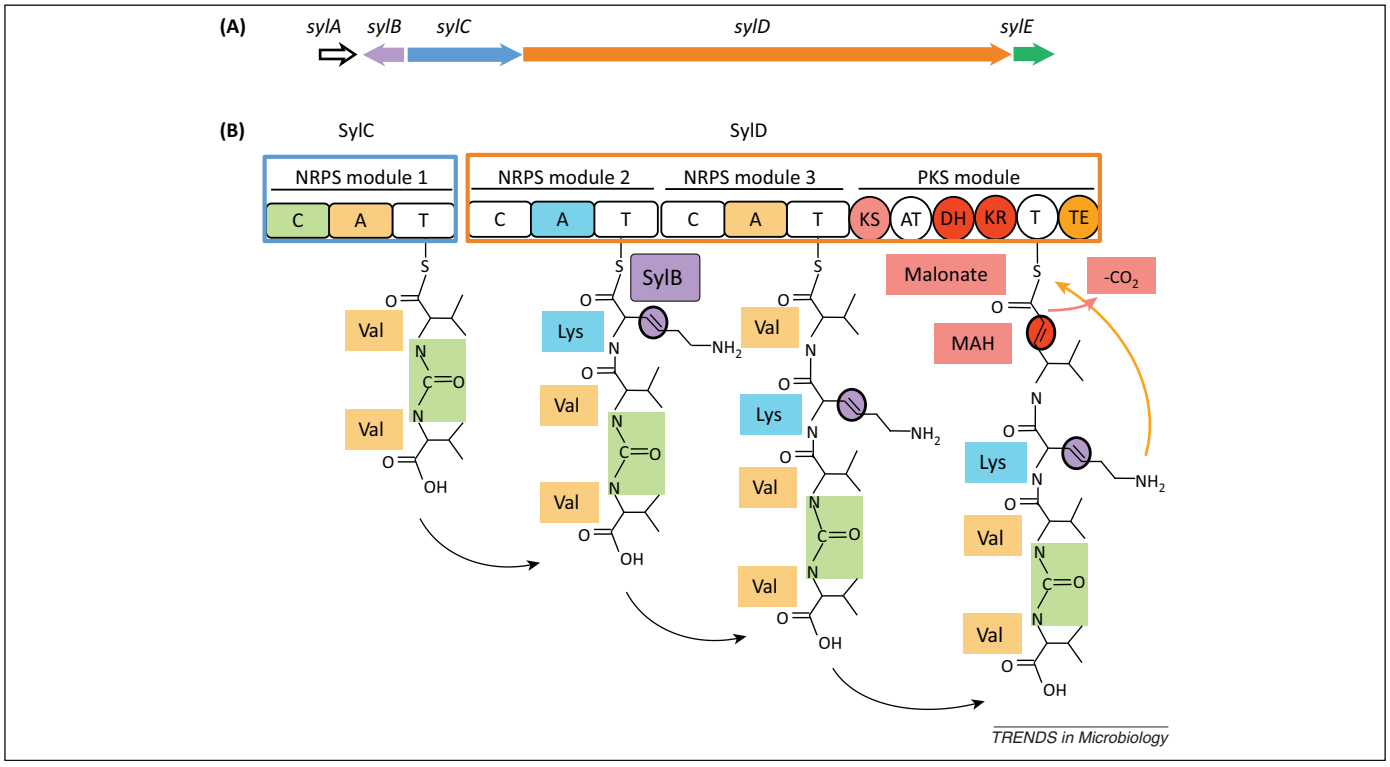


Figure 2. Syringolin A (SylA) biosynthesis. **(A)** Gene cluster encoding the SylA synthetase of *Pseudomonas syringae* pv. *syringae* (*Pss*) strains. The *sylA* gene encodes a LuxR-type transcriptional activator regulating transcription of the *sylB* gene and the *sylCDE* operon [55], whereas the *sylE* gene encodes an exporter belonging to the major facilitator superfamily. The *sylC* and *sylD* genes encode the non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules, which, together with the *sylB* gene product, compose the SylA synthetase. **(B)** SylA biosynthesis model. The SylA synthetase contains three NRPS modules with condensation (C), adenylation (A), and thiolation (T) domains and a type 1 PKS module with ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), T, and thioesterase (TE) domains. The SylC NRPS starter module activates valine (Val) and *N*-acylates it with a second valine, thus forming an ureido linkage by incorporation of bicarbonate/CO_2 [56,57]. The first NRPS module of SylD is thought to activate lysine (Lys), which is desaturated to 3,4-dehydrolysine by the *sylB* gene product [43]. The second SylD NRPS module is thought to activate Val, which, after condensation/decarboxylation to a malonate residue activated by the type I PKS module of SylD, results in a β-keto-thioester whose β-keto group is twofold reduced by the KR and DH domains, thus resulting in the 5-methyl-4-amino-2-hexenoic acid (MAH) moiety. Finally, lactam bond formation between the terminal carboxyl group and the ε-amino group of the 3,4-dehydrolysine moiety catalyzed by the TE domain of the PKS module leads to cyclization and release of the final product [58]. Genes, domains/proteins, and the molecular entities they act upon are given in the same color shades. Adapted, with permission, from [54].

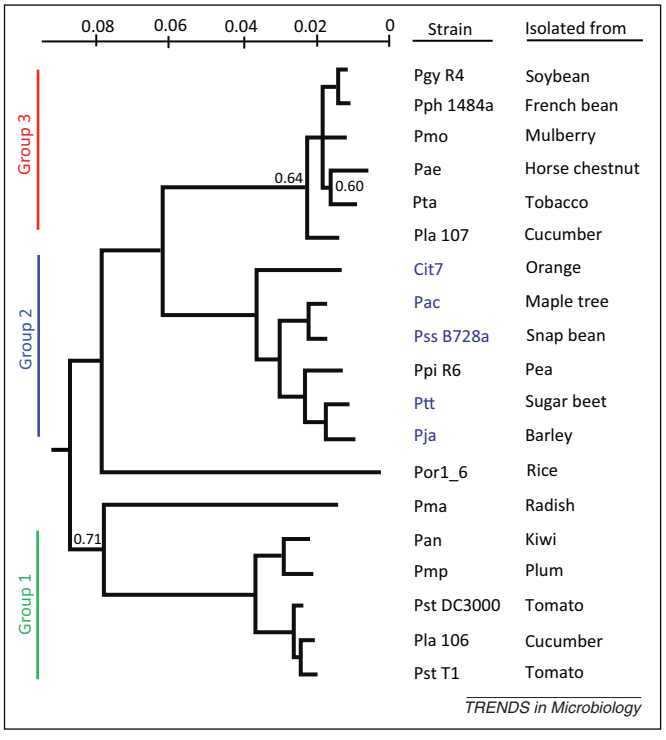


Figure 3. Bayesian phylogenetic tree of 19 *Pseudomonas syringae* strains. Colored bars on the left indicate phylogenetic groups as originally defined in [74]. Strains labeled in blue contain complete *syl* gene clusters. Pathovar of strain designations (if not given in the text): Pgy, *glycinea*; Pph, *phaseolicola*; Pmo, *mori*; Pae, *aesculi*; Pta, *tabaci*; Pla, *lachrymans*; Cit, unknown; Pac, *aceris*; Ppi, *pisi*; Ptt, *aptata*; Pja, *japonica*; Por, *oryzae*; Pma, *maculicola*; Pan, *actinidiae*; Pmp, *morsprunorum*. Adapted, with permission, from [59].

syl gene clusters with very high sequence conservation at conserved positions in the genomes of five of six strains belonging to group (subclade) 2, but not in strains belonging to groups 1 and 3, suggesting that SylA biosynthesis may be restricted to group 2 strains (Figure 3) [59]. Interestingly, group 2 strains seem to encode fewer T3Es (9 to 16) than strains of the other groups. The comparatively low numbers of T3Es encoded in the analyzed group 2 strains, which correlates with the presence genes for the biosynthesis of the phytotoxins syringopeptin and syringomycin, as well as of syringolin, suggest a virulence strategy distinct from the one of group 1 (which encompasses *Pst* DC3000) and group 3 strains [59].

Conflicting action of virulence factors?

Concomitant production of proteasome inhibitors with virulence factors requiring the host proteasome for their activity seems contradictory. In *X. campestris* pv. *vesicatoria*, the T3Es XopJ (proteasome inhibitor) and XopL (an E3 ligase) represent such a pair. The seeming conflict might be mitigated by spatial and/or temporal separation

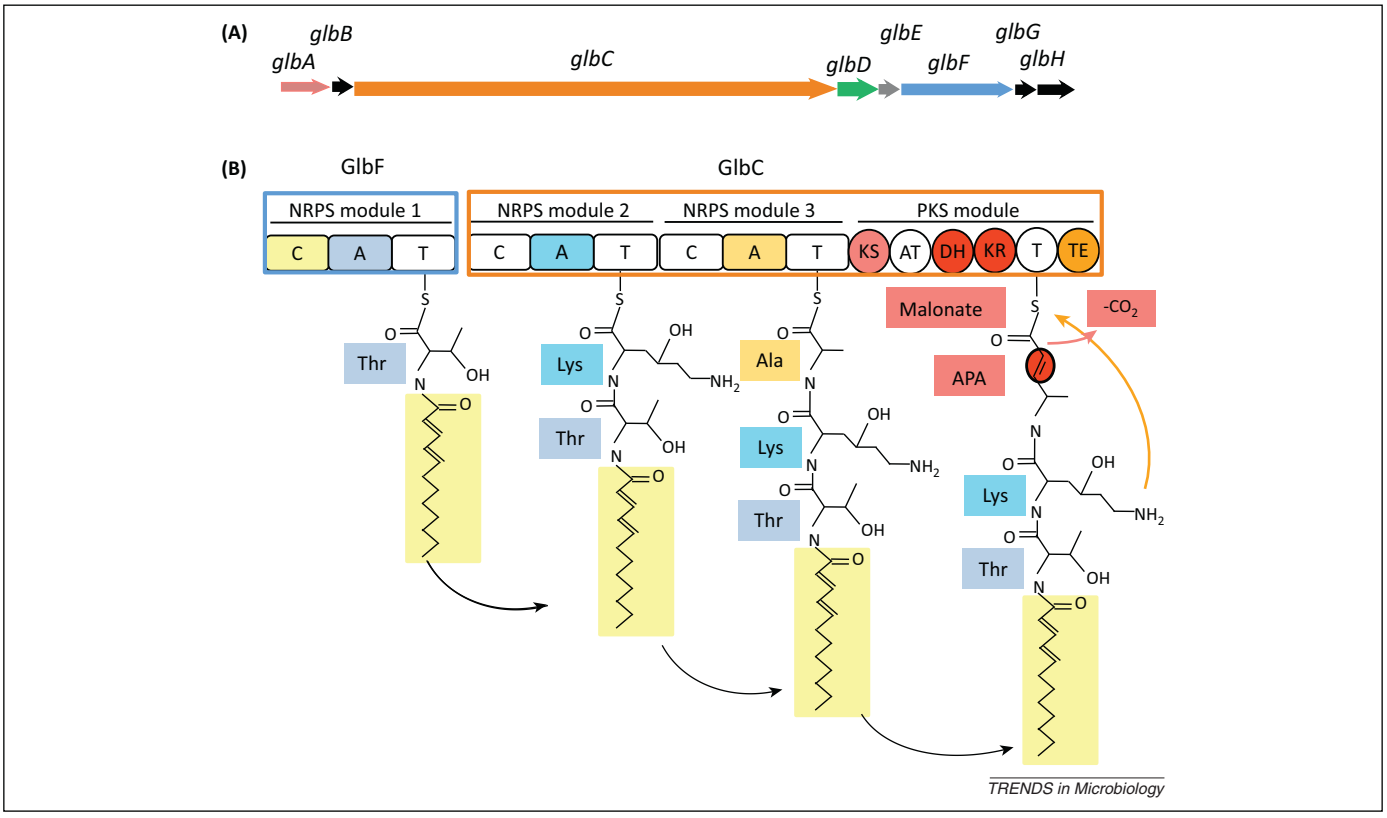


Figure 4. Glidobactin A (GlbA) biosynthesis. **(A)** The GlbA synthetase gene cluster. The *glbC, glbD,* and *glbF* genes are shown in the same colors as their *syl* homologs in Figure 2. The *glbA* gene encodes an LysR-type transcriptional regulator [64], whereas *glbE* encodes an MbtH-like chaperone [65]. The function of the *glbB, glbG,* and *glbH* genes is currently unknown. **(B)** GlbA biosynthesis model. The GlbF non-ribosomal peptide synthetase (NRPS) starter module activates threonine (Thr) and *N*-acylates it with a coenzyme A-activated fatty acid [65]. The first NRPS module of GlbC is thought to activate lysine (Lys), which is oxidized to 4-hydroxylysine by an as yet unknown enzyme. The second NRPS module is thought to activate alanine (Ala), which, after condensation/decarboxylation to a malonate residue activated by the type I polyketide synthase (PKS) module of GlbC, results in a diketide that is twofold reduced by the ketoreductase (KR) and dehydratase (DH) domains, thus resulting in the 4-amino-2-pentenoic acid (APA) moiety. Macrolactam ring formation and release is catalyzed by the thioesterase (TE) domain. Genes, domains/proteins, and the molecular entities they act upon are given in identical color shades. Adapted, with permission, from [64].

of production or activities [38]. In *P. syringae*, SylA production would seem to counteract host proteasome-requiring T3Es such as HopAB2 and HopM1 or other virulence factors such as the JA-mimicking small-molecule coronatine (COR). To our knowledge, COR synthetase genes or COR biosynthesis so far have not been found in strains carrying SylA synthetase genes [59,60]. A homolog of HopAB2 (HopAB1, alias $AvrPtoB_{B728a}$) occurs in the SylA-producing strain *Pss* B728a, which, however, has recently been shown to be defective in its E3 ligase activity [61]. In the case of HopM1, a variant of which occurs in *Pss* B728a as well as in most *P. syringae* strains, it is also not clear whether it has a proteasome-dependent function related to the one of $HopM1_{DC3000}$. HopM1 variants are phylogenetically and functionally diverse as evidenced by the fact that a variant from *P. syringae* pv. *morspunorum* did not complement a $hopM1_{DC3000}$ deletion mutant [59]. As mentioned above, if the potential to produce conflicting virulence factors does occur in the same strain, conflict might be resolved by differential regulation of biosynthesis during host colonization.

Syrbactins in animal pathogens

The determination of the structure of SylA revealed its similarity to the glidobactins (similar to cepafungins), a family of agents from soil bacteria with antitumor and antifungal activity isolated more than 20 years ago [62,63]. Similar to SylA, glidobactin A (GlbA) contains a 12-membered macrolactam ring with its characteristic α,β-unsaturated carbonyl group (Figure 1). GlbA was shown to inhibit the ChTL and TL activities of the eukaryotic proteasome by the same mechanism as SylA, whereas the CL activity was not inhibited at all [44]. The GlbA synthetase gene cluster (*glbA–glbH*) was cloned from the soil bacterium K481-B101, which belongs to the Burkholderiales [64]. Sequence and architecture of *glbC* and *glbF*, which, similar to their homologs *sylD* and *sylC* in *Pss*, encode a mixed NRPS/PKS synthetase, allowed to postulate a GlbA biosynthesis model which is analogous to the one for SylA, and is experimentally supported [64,65] (Figure 4).

Whereas disrupted nonfunctional *glb*-like gene clusters are present in strains of *Burkholderia mallei* (causing glanders in donkeys and horses), apparently intact *glb*-like gene clusters were found in the genome sequences of the human pathogens *Burkholderia pseudomallei* (the causing agent of melioidosis, β-proteobacteria), *Burkholderia oklahomensis* (β-proteobacteria), and *Photorhabdus asymbiotica* (γ-proteobacteria). In addition, an intact *glb*-like gene cluster is also present in the insect pathogen and nematode symbiont *Photorhabdus luminescens*. This suggests that these pathogens have the potential to synthesize syrbactins (Figure 5) [64]. Indeed, transfer of the *glb*-like gene cluster of *Ph. luminescens* ssp. *laumondii* TT01 into *Pseudomonas putida* resulted in GlbA production in the

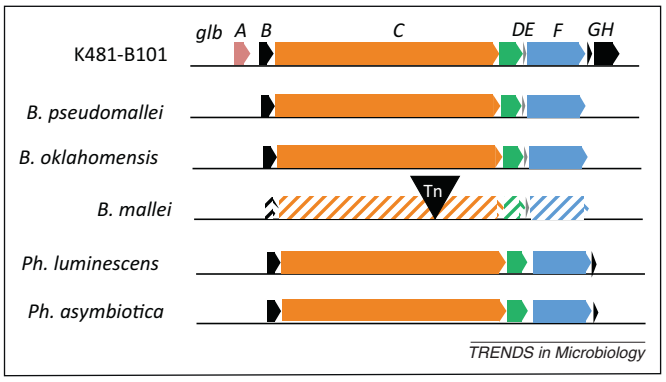


Figure 5. Glidobactin A (GlbA)-like synthetase gene clusters in animal pathogens. Homologous genes are shown in identical colors. Hatched *Burkholderia mallei* genes indicate nonfunctionality due to transposon (Tn) insertions and additional strain-specific mutations and rearrangements. Accession numbers of proteins encoded by functional gene clusters are as follows: K481-B101, CAL80819–CAL80826; *Burkholderia pseudomallei* K96243, YP_111277–YP_111273; *Burkholderia oklahomensis* C6786, WP_010109152–WP_010109148; *Photorhabdus luminescens* ssp. *luminescens* TT01, NP_929149–NP_929145; *Photorhabdus asymbiotica* ssp. *asymbiotica* (ATCC 43949), CAR67624–CAR67620. Adapted, with permission, from [64].

latter species [66]. Furthermore, GlbA as well as a variant named cepafungin I, the strongest proteasome inhibitor known to date, were isolated from *Ph. luminescens* cultures [67]. Similarly, GlbA and derivatives named luminmycins have been isolated from *Ph. asymbiotica* cultures and from crickets after infection with the pathogen [68]. Thus, these pathogens indeed produce syrbactin-type proteasome inhibitors, although future research must elucidate whether and how they are involved in virulence.

Evolution of syrbactins

Syrbactin synthetase-encoding genes so far have been found in a small but diverse group of plant and animal pathogens, suggesting that they were acquired by horizontal gene transfer. Comparison of sequence and architecture of the genes encoding the NRPS/PKS synthetase modules within *syl* and *glb* gene clusters reveals that the location of the starter module genes *sylC* and *glbF* is not conserved (Figures 2 and 3). In addition, and in contrast to *sylD*, whose closest homologs found in genome databases are the *glbC*-like genes, the closest homologs of the *sylC* gene are not the *glbF*-like genes. This suggests that *sylD* and *glbC* are descendants of a common ancestral gene, whereas *sylC* and *glbF* were independently recruited into the SylA- and GlbA-like synthetase gene clusters, respectively. The starter modules not only activate different amino acids but also catalyze the addition of tails with chemically different characters [56,65]: in the case of SylA it is an ureido valine, leading to a hydrophilic compound, whereas in GlbA it is a fatty acid, rendering the compound much more hydrophobic (Figure 1). This difference determines whether and how syrbactins are taken up into cells. GlbA has antifungal activity [62] and evidently must be taken up by fungal cells, such as yeast, whereas yeast is completely insensitive to SylA [46]. However, yeast can be rendered sensitive towards SylA by expression of the *A. thaliana* YLS7 and YSL8 plant-specific transporters [46]. Thus, the different tails may be viewed as adaptations to kingdom-specific import mechanisms.

Box 2. Outstanding questions

- How prevalent is proteasome inhibition as a virulence strategy in pathogens?
- How widespread is the production of syrbactin-type proteasome inhibitors in plant and animal pathogens and in microbes in general?
- What is the function of syrbactin-type proteasome inhibitors in animal and human pathogens? Do they also enhance virulence? Could they have a function in symbiosis (e.g., in *Photorhabdus luminescens*)?
- In addition to XopJ of *Xanthomonas campestris*, are other type III effectors, both within and outside the YopJ family, interfering with proteasome function?

Concluding remarks

Whereas an increasing number of T3Es in both plant and animal pathogens have been found to require the host UPS for their mode of action, proteasome inhibition as a (potential) virulence strategy has only recently become apparent in a small but interesting group of plant and animal pathogens. This group will without doubt grow in the future, but it remains to be seen how prevalent proteasome inhibition as a virulence strategy of pathogens is. In view of the involvement of the UPS in regulatory pathways controlling almost all aspects of plant life, proteasome inhibition appears, due to the expected pleiotropic consequences, rather like a drastic sledgehammer method to suppress plant immunity. This contrasts with the often exquisitely precise interference with specific host defense signal transduction components that characterize the action of many T3Es. However, this view may be exaggerated. Tight regulation of inhibitor production [55], the inhibitor-triggered *de novo* synthesis of proteasomes as suggested by transcriptome analyses [69], as well as spatial restriction of the inhibitor to the neighborhood of infection sites due to cellular uptake and proteasome binding may render proteasome inhibition much less pleiotropic than suggested by the known involvement of the UPS in a plethora of cellular processes. This is evidenced by the fact that SylA is not a deadly toxin that kills cells but helps biotrophic bacteria to suppress the SA-dependent defense pathway and to spread from wound infection sites [52,53]. Further research (Box 2) will be required to determine the contribution of syrbactins and other proteasome inhibitors to the virulence of pathogens.

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Review

The role of aquatic ecosystems as reservoirs of antibiotic resistance

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Although antibiotic resistance has become a major threat to human health worldwide, this phenomenon has been largely overlooked in studies in environmental settings. Aquatic environments may provide an ideal setting for the acquisition and dissemination of antibiotic resistance, because they are frequently impacted by anthropogenic activities. This review focuses primarily on the emergence and dissemination of antibiotic resistance in the aquatic environment, with a special emphasis on the role of antibiotic resistance genes.

Antibiotic resistance: emergence and impact

The development of antibiotics has been one of the major achievements of the 20th century and millions of human lives have been saved since the 1940s when the first antibiotics, penicillin and streptomycin, were introduced. Antibiotics are used to treat a wide range of bacterial infections and are indispensable in medical treatment such as intensive care, organ transplantation, chemotherapy, care of preterm babies, and surgical procedures, which could not be performed effectively without the availability of effective antibiotics.

However, antibiotic resistance has become a global public health concern because the organisms that cause infections are becoming resistant to the most commonly prescribed antibiotic treatments, resulting in prolonged illness and greater risk of death [1]. Another worrying aspect is that antibiotic resistance has developed over time, from resistance to single classes of antibiotics to multidrug resistance and extreme drug resistance [2], increasing the challenge for the development of more effective antibiotics. According to recent data from the European Centre for Disease Prevention and Control and the European Medicines Agency, every year approximately 25 000 European citizens (5.1 per 100 000 inhabitants) die from infections caused by bacteria that have developed resistance towards antimicrobials (http://www.ecdc.europa.eu/). In the USA, nosocomial infections are responsible for 12 000 deaths (4.0 per 100 000 inhabitants) each year [3], and it is estimated that more than 70% of bacteria that cause these infections are resistant to at least one of the antibiotics commonly used to treat them [4].

Susceptible bacteria may become resistant to antibiotics through multiple and complex mechanisms, such as (i) exclusion of the antibiotic by the cell membrane; (ii) intracellular modification and/or deactivation of the antibiotic; (iii) reduction in sensitivity of the cellular target; (iv) extrusion from the cell; and (v) intracellular sequestration [5]. These mechanisms can evolve through mutation and selection or by acquiring from other bacteria the genetic information that encodes resistance. The last event may be mediated by horizontal gene transfer (HGT), which is largely, although not exclusively, responsible for the development of antibiotic-resistant bacteria through various processes such as conjugation (by bacterial plasmids and conjugative transposons), transformation (by acquisition of free naked DNA from the environment), and transduction (by bacteriophages).

Despite the fact that antibiotic resistance is a major and growing public health concern, the surveillance for the expansion of this phenomenon in environmental settings is remarkably limited. One possible explanation could be the fact that antibiotic concentrations in nonclinical settings are generally very low. However, recent studies have revealed that selection of resistant bacteria can occur at extremely low antibiotic concentrations [6], similar to those concentrations found in some aquatic and soil environments [7,8], showing that even subinhibitory concentrations of antibiotics may promote antibiotic resistance. Moreover, the overuse and misuse of antimicrobial agents in human and veterinary medicine, animal farming, industrial settings, and their subsequent release in wastewater treatment plants (WWTPs) have contributed to the emergence and dissemination of resistant bacteria into the environment, including bacteria causing infections in both humans and animals [9–11]. Given this, aquatic environments including surface water and groundwater bodies provide ideal settings for the horizontal exchange of mobile genetic elements (MGEs) encoding antibiotic resistance [5,12]. This review will, therefore, focus on the emergence and dissemination of antibiotic resistance in the aquatic environment, with a special emphasis on the role of antibiotic resistance genes (ARGs).

Link between clinical and environmental resistance

Several studies suggest that antibiotic resistance occurs in nature and has an ancient origin, which is not linked to the

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anthropogenic use of antibiotics [13,14]. In the environment, bacteria have developed an ability to synthesize bioactive molecules to either cooperate with or antagonize other members of the community and, as a result, they have developed defense systems to protect themselves against the molecules of others. These molecules are encoded by genetic elements which constitute the resistome [15]. Moreover, the new high-throughput sequencing tools have revealed that an 'intrinsic resistome' exists, including innumerable sequences normally belonging to bacterial metabolic networks that can eventually participate in resistance to antimicrobial agents. These pre-resistance genes can evolve to new resistance mechanisms if they reach an environment with a high concentration of antibiotics. In this regard, antibiotics may act as selector agents of mechanisms of resistance but also as accelerator agents of the evolution of resistance [16]. Several metagenomic studies have been performed to explore antibiotic resistance diversity in different environments. In fact, one study demonstrated that uncultured soil bacteria from a Wisconsin oak savannah harbored unknown aminoglycoside and tetracycline resistance genes significantly diverse from previously sequenced genes [17]. Another study found genes encoding for β-lactamases in one Alaskan soil, where the anthropogenic activities are minimal. These β-lactamases were more closely related to ancestral β-lactamases than those isolated in clinical settings but they were still capable of conferring resistance on *Escherichia coli* [18].

Collectively, these studies suggest that the environment represents a huge reservoir of ARGs; however, only within the past 10 years has there been evidence of the mobilization of environmental resistance genes into clinical human pathogens. Some studies, for instance, have shown the similarity of the gene encoding for CTX-M, an extended spectrum β-lactamase (ESBL) often located in clinical pathogens, with chromosomally encoded β-lactamases from *Kluyvera* spp., a typical environmental bacterium [19–21]. It has also been demonstrated that genes encoding quinolone resistance may be present in environmental bacteria. The origin of the *qnrA* gene, which confers low levels of resistance to quinolones, was identified in the chromosome of *Shewanella algae*, a Gram-negative species widely distributed in marine and freshwater environments [22]. The reservoirs of the *qnrB* and *qnrS* genes remain unknown, although it seems that these genes may be closely related to chromosome-encoded Qnr-like determinants in some species of the *Vibrionaceae* family [23]. More recently, a platform has been developed to facilitate the rapid and efficient functional characterization of metagenomic libraries, namely Parallel Annotation and Reassembly of Functional Metagenomic Selections (PARFuMS). Applying this platform to a collection of soil-derived cultures, ARGs of all major mechanistic classes were found in nonpathogenic soil bacteria with perfect nucleotide identity to ARGs from many diverse human pathogens. Moreover, the ARGs were located within long sequences flanked by MGEs, suggesting a recent HGT event and probably the mechanism through which this exchange occurred [24].

Now that the link between clinical and environmental resistance has been demonstrated, it is essential to study how antibiotic resistance evolves in the environment (Box 1). Moreover, owing to the introduction of antibiotics into the environment from human and veterinary applications, the environment turns into a reactor where bacteria from different origins, antibiotics, disinfectants, and heavy metals are mixed, contributing to the evolution and dissemination of antibiotic resistance [25]. Wright *et al.* [26] quantified the abundance of an MGE, the class 1 integrase (*intI1*) gene, in total community DNA extracted from contaminated and reference riverine and estuarine microhabitats, and in metal- or antibiotic-amended freshwater microcosms. The authors found that the *intI1* gene was more abundant in all contaminant-exposed bacterial communities indicating that relative gene transfer potential is higher in these communities. Likewise, Rosewarne *et al.* [27] demonstrated that the abundance of *intI1* was increased as a result of ecosystem perturbation, indicated by a strong positive correlation with heavy metals such as zinc, mercury, lead, and copper. Both studies suggest that the presence of some pollutants, such as heavy metals, could co-select for antibiotic resistance.

Although a high number of ARGs have been found in environmental settings influenced by anthropogenic activities, these increases remain partially understood. There are two main hypotheses to explain these observations. The first suggests that antibiotics released in the environment exert a selection pressure on bacteria, selecting the resistant populations and thus increasing the amount of ARGs. The second hypothesis proposes that ARGs from other sources, such as human and animal origin, are transported mostly through runoff processes into the aquatic environment [28]. A study carried out in the South Platte River basin supports the hypothesis of transport, because the molecular signatures between pristine and impacted sites were different and because ARGs were detected with greater frequency in suspended sediments than in streambed sediments, where the antibiotic concentrations were higher [29]. Conversely, a metagenomic study was performed to investigate how microbial communities respond to the presence of wastewater discharges. The results revealed high levels of ARGs as well as elements for HGT, which suggest that those discharges carrying antibiotic residues promote ARGs and the exchange of MGEs [30]. Another study revealed that repeated applications of manure increase the abundance of ARGs in soil, suggesting that HGT is an important factor in the dissemination of ARGs, because bacteria from manure may not be well adapted to the soil environment [31]. Ultimately, the natural environment, either pristine or polluted, possesses a pool of ARGs that should be taken into account to better understand the evolution and dissemination of antibiotic resistance.

Acquisition and dissemination of ARGs

Several studies have demonstrated that ARGs are spread by MGEs, including plasmids, insertion sequences, insertion sequence common region elements, transposons, integrons, genomic islands, integrating conjugative elements, and bacteriophages (Table 1, [32–38]), which are involved in bacterial acquisition and recombination of foreign DNA [39]. Plasmids, circular double-stranded DNA molecules

Box 1. Further research on antibiotic resistance in the aquatic environment

The high efficiency of MGEs transferring ARGs among phylogenetically distant bacteria from different environments makes it difficult to distinguish between naturally occurring resistance and the resistance promoted by antibiotics released from anthropogenic sources. However, the European Council concluded in 1998 that there was a relationship between the consumption of antimicrobial compounds and the prevalence of antibiotic-resistant bacteria. Since then, some government agencies have launched several programs for antibiotic resistance surveillance to assess the actual risk for public health associated with the consumption of antimicrobial compounds. Nevertheless, recent advances in metagenomics have shown that very little is known about the antibiotic resistome of the vast majority of environmental bacteria. Antibiotic resistance is by far better understood at the small scale (i.e., within an individual bacterium) than at the large scale (the natural environment). Given that antibiotics are the main weapon against bacterial pathogens, monitoring and surveillance programs and further research of environmental reservoirs is needed to better understand the mechanisms of antibiotic resistance and counteract this major, growing problem.

that replicate independently from chromosomal DNA, may encode a wide variety of genetic determinants. Insertion sequences (ISs) are the smallest and simplest autonomous MGEs, which possess one or two open reading frames and encode a transposase surrounded by linker regions that frequently end with short terminal inverted repeat sequences. Insertion sequence common region (IS*CR*) elements, which differ from the ISs by lacking terminal inverted repeat sequences, are thought to transpose by a mechanism termed rolling circle transposition. Transposons are gene systems flanked by inverted repeat sequences and encode transposases, which specifically recognize and introduce nicks at the ends of these elements to allow for integration at ISs. Integrons possess a site-specific recombination system for the capture of genes, notably those encoding antibiotic resistance, that are contained within mobile gene cassettes. Genomic islands are large DNA segments present in most bacterial genomes that have been acquired by HGT and often contain blocks of genes offering a selective advantage for host bacteria. Finally, integrating conjugative elements (ICEs) are self-transmissible MGEs that integrate into and replicate along with the host cell chromosome. The role of these MGEs as efficient vehicles for HGT has been previously described in detail [40–44]; however, the contribution of bacteriophages to the dissemination of ARGs has not been extensively explored in environmental settings.

Bacteriophages, also known as phages, are viruses that infect bacteria in order to replicate and assemble themselves using the metabolism and machinery of the host cell. Some phages may act as mediators of HGT, through transduction, whereby DNA is transferred from one bacterial cell to another by a phage. Transduction can be generalized, when fragments of bacterial DNA are packaged at random in the phage particles, or specialized, where certain regions of bacterial DNA are carried along with the viral genome [45].

Phages are considered to be the most abundant known organisms, and have a powerful influence on bacterial community structure and function through predation and gene transfer [46]. The relatively recent introduction of molecular techniques has led to a greater understanding of microbial diversity and its role in nature. A recent study revealed the presence of β-lactamases and putative transposases in the phage metagenome of activated sludge [47]. Interestingly, Colomer-Lluch *et al*. [38] determined the presence of ARGs conferring resistance to β-lactams and methicillin in phage DNA from a WWTP and water samples of the receiving river. The authors also demonstrated that ARGs from phage DNA were transferred to susceptible strains, which became resistant to ampicillin. ARGs have also been observed to confer resistance to β-lactams and quinolones in phage DNA from different hospital and WWTP effluents [48]. Altogether, these results undoubtedly demonstrate the contribution of phages in the dissemination of ARGs into the environment.

Aquatic environments as reservoirs of ARGs

It is now clearer than ever that antibiotics act as promoters of antibiotic resistance, and thus may contribute to the

Table 1. Summary of MGEs found in prokaryotes

Genetic element	Characteristics	Examples of resistance determinants	Refs
Plasmid	Variable size (1 to >100 kb), self-transmissible or mobilizable	Plasmid pP2G1 contains ARGs, which confer resistance to different antibiotics	[32]
Insertion sequence	Small (<2.5 kb), contains terminal inverted repeats, and encodes a transposase	IS18 mediates overexpression of the $bla_{OXA-257}$ gene	[33]
IS*CR* elements	Transpose adjacent DNA sequences by rolling circle transposition	IS*CR1* mediates the mobilization of the $bla_{CTX-M-1}$ gene	[34]
Transposon	Large (<12 kb), flanked by IS or inverted repeats; encodes a transposase and other functional genes such as ARGs	Tn*1* and Tn*3* confer resistance to β-lactams	[35]
Integron	Mediates the capture and expression of gene cassettes; encodes for an integrase, attachments sites, and transcriptional elements	Class 1 contains different cassettes conferring multidrug resistance	[32]
Genomic island	Large mobile regions of DNA that encode complex biological functions	SGI1 confers resistance to streptomycin, spectinomycin, β-lactams, and sulfonamides	[36]
Integrating conjugative elements	MGEs that are integrated in the chromosome and transfer via conjugation	ICE*Vch*Hai1 contains different ARGs	[37]
Bacteriophages	Viruses that infect prokaryotic cells; their replication allows the DNA transfer from one bacterium to another, known as transduction	β-Lactamase genes have been isolated from environmental phages	[38]

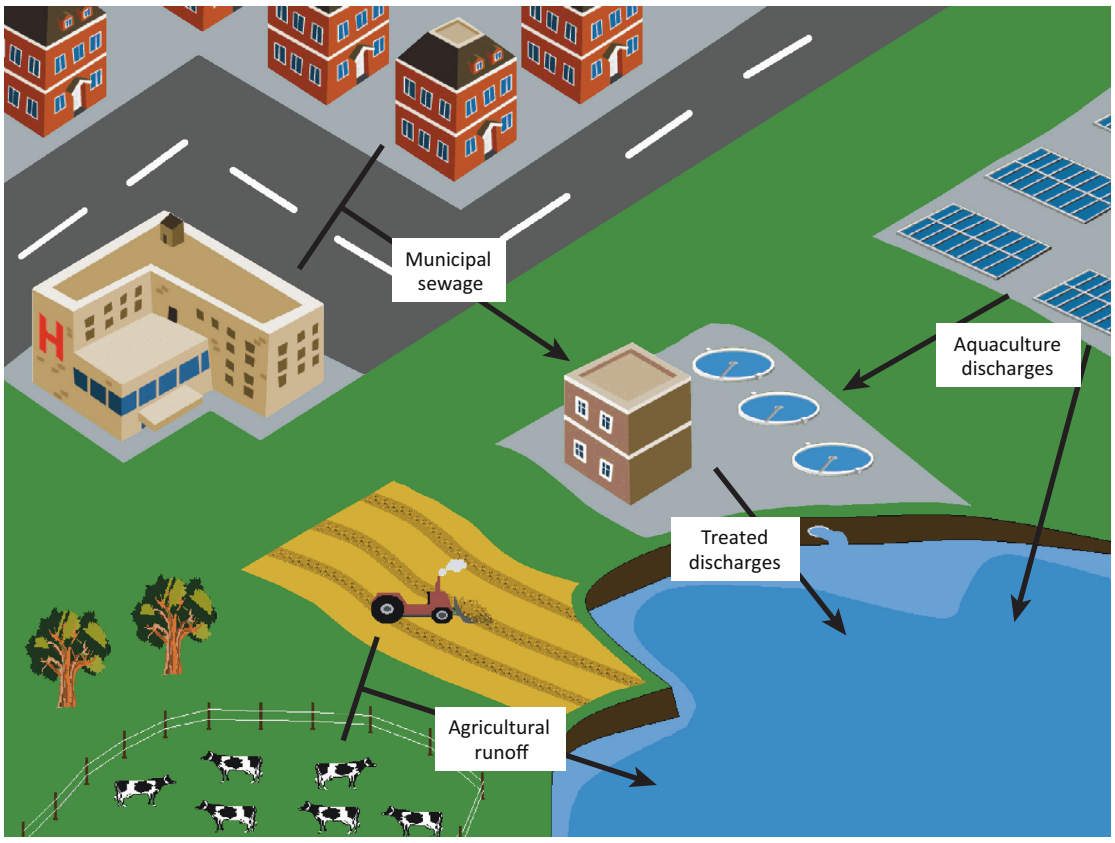


Figure 1. Different anthropogenic activities that result in the dissemination of antibiotic resistance genes (ARGs) in aquatic environments.

acquisition of MGEs [49]. As mentioned above, a diverse mixture of antibiotics and other pollutants, their metabolites and resistant bacteria, reaches the aquatic environment through treated and untreated sewage, hospital waste, aquaculture discharges, and agricultural runoff (Figure 1). These aquatic compartments, such as water and sediment, may therefore have a significant role in driving ARG transfer, ecology, and evolution [5].

WWTPs represent the main sites through which antibiotics are released into the environment. Some studies have suggested that common technologies that are being applied in WWTPs, such as biological treatment, provide an ideal environment (i.e., high bacterial densities, high oxygen, and high nutrient concentrations) for HGT, because bacteria are in continuous direct contact with antibiotics and resistant bacteria [50]. A metagenomic study recently found 140 different ARGs in bacteria from activated sludge and 123 ARGs in the final effluent of a WWTP [51]. Moreover, LaPara *et al*. [52] investigated the impact of tertiary treated municipal wastewater on the quantity of several ARGs in Duluth–Superior Harbor and determined that the treated water was a significant point source of three tetracycline resistance determinants as well as the *intI1* gene into the receiving harbor. We also investigated the effect of WWTP effluents on the prevalence of several ARGs in the Ter River and observed a significant increase in the relative abundance of ARGs in biofilm samples collected downstream of the WWTP discharge [53]. These studies suggest that WWTP discharges could promote the dissemination of ARGs into the environment.

The majority of bacteria in natural aquatic ecosystems are organized in biofilms, which facilitate their survival and dispersal. Biofilms are aggregations of bacteria that live in a highly structured and organized community. Recent studies have suggested that biofilms may contribute to antibiotic resistance, and this may be due to high cell density, close proximity, and accumulation of MGEs. Schwartz *et al*. [54] demonstrated that the *vanA* gene, which confers high-level resistance to vancomycin, was detected not only in wastewater biofilms but also in drinking water biofilms in the absence of enterococci, suggesting possible gene transfer to autochthonous drinking water bacteria. Engemann *et al*. [55] found evidence that the abundance of tetracycline resistance genes was reduced at different rates in the water column, and some genes readily migrated into biofilms, which suggest that biofilms serve as long-term reservoirs for ARGs.

Aquatic sediments also represent an important environmental matrix within which genetic transfer and recombination occur. Antibiotics used in aquaculture and other anthropogenic activities may be retained in these sediments, which can act as an interface for a complex and dynamic community of microorganisms, facilitating the transfer, maintenance, and dissemination of MGEs. Kristiansson *et al*. [30] demonstrated that MGEs, such as class 1 integrons and IS*CR* elements, were highly overrepresented in river sediments exposed to antibiotics. Moreover,

Cummings *et al*. [56] detected five different plasmid-mediated quinolone resistance (PMQR) determinants (including *qnrA*, *qnrB*, *qnrS*, *qepA*, and *aac(6′)-Ib-cr*) in surface sediments from a sewage-impacted coastal wetland along the US–Mexico border, whereas sediments of a nearby urban wetland that was largely unaffected by sewage contained only three different PMQR determinants (*qnrB*, *qnrS*, and *qepA*). Likewise, Chen *et al*. [57] studied the accumulation of ARGs in surface and bottom waters as well as sediments from the Pearl River estuary. The authors found that concentrations of tetracycline resistance genes in the sediments were at least 100 times higher than those in the water, and their concentrations depended on the degree of anthropogenic impacts from the river to the coast. Yang *et al*. [58] investigated the resistome from marine sediments using high-throughput sequencing and metagenomic analysis. They observed that several contigs shared high identity with transposons or plasmids from human pathogens. Together with biofilms and sediments, aquatic organisms may also be important intermediates in the development and dissemination of ARGs [5]. Meibom *et al*. [59] suggested that chitin, an important structural component of many crustaceans and mollusks, not only serves as a surface for biofilm formation in aquatic environments but it also induces competence for natural transformation. The authors demonstrated that competence was experimentally mediated by the acquisition of ARGs during growth of *Vibrio cholerae* on crab shell fragment immersed in seawater. Jiang *et al*. [60] recently demonstrated high levels of ARGs, including ESBLs and PMQR determinants, in bacterial strains isolated from fish intestinal samples in China. Interestingly, Oguri *et al*. [61] observed that ciliates promote the transfer of genes encoding ESBLs among *E. coli* strains, suggesting that the presence of these organisms, which are widely distributed in nature, may provide gene exchange among bacterial populations from different environments. Additionally, several environmental factors, such as temperature, may affect HGT and thereby delineate exchange community boundaries [62]. Walsh *et al*. [63] isolated some $bla_{\text{NDM-1}}$-positive strains from water samples and observed that the highest frequency of conjugation between cells in suspension occurred at 30 °C compared with 25 °C and 37 °C, which suggests that gene transfer may be more frequent in geographic areas with higher temperatures.

Concluding remarks

With the increasing use and accessibility to culture-independent techniques that allow the functional and structural characterization of microbial communities, the exploration of ARGs may extend to a wide range of organisms, including uncultured species. These advances will increase our understanding of the evolutionary pathways through which the MGEs encoding ARGs spread within a community. Taking into consideration that ARGs are 'easy to get, hard to lose' pollutants and that they may be detected in antibiotic-free environments or even when the selective pressure has disappeared, the scientific community and public authorities should decisively collaborate for the implementation of strategies, policies, and programs that will limit the use of antibiotics, and thus the development and dissemination of ARGs in clinical and environmental settings.

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Review

The emergence of *mecC* methicillin-resistant *Staphylococcus aureus*

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The report of methicillin-resistant *Staphylococcus aureus* (MRSA) encoding a divergent *mecA* gene in 2011 was highly significant. This homologue, designated *mecC*, poses diagnostic problems with the potential to be misdiagnosed as methicillin-sensitive *S. aureus*, with important potential consequences for individual patients and for the surveillance of MRSA. *mecC* MRSA have now been reported from 13 European countries and have been isolated from 14 different host species, with evidence of a recent increase in Denmark. The emergence of *mecC* MRSA is a topic of interest to human and veterinary microbiology, and we consider it timely to review here its discovery and subsequent investigation.

Staphylococcus aureus and MRSA in humans and animals

S. aureus is a prominent human pathogen that can cause a diverse range of diseases ranging from relatively minor skin infections to serious and life-threatening infections such as endocarditis, pneumonia, and sepsis. Its impact is enhanced by the development of antibiotic resistance, most notably methicillin-resistant *S. aureus* (MRSA) that is resistant to virtually all β-lactam antibiotics. Although originally regarded as a nosocomial pathogen (hospital-associated MRSA or HA-MRSA), MRSA infections among previously healthy individuals in the community, without links to healthcare settings, emerged in the 1990s and are referred to as community-associated MRSA (CA-MRSA). For the most part HA-MRSA and CA-MRSA involve different lineages, but these distinctions are not absolute, and transfer of strains between these settings is increasingly recognised. In addition to its importance as a human pathogen, *S. aureus* [1], including MRSA [2,3], can colonise and infect a wide range of host species including livestock, wildlife, and companion animals, with bovine mastitis among dairy cattle, lameness in poultry, and severe and lethal infections in farmed rabbits being particularly significant in terms of economic impact. MRSA in animals is not only important from an animal welfare and economic perspective but can act as a reservoir for zoonotic infection of humans. In particular, multilocus sequence type clonal complex (CC)398 is abundant among pigs and other livestock in mainland Europe, and infection of humans in close contact with these animals has led to the recognition of a third epidemiological form of MRSA, livestock-associated MRSA (LA-MRSA) [4].

Mechanism of methicillin-resistance in MRSA and its diagnostic detection

Although methicillin is no longer produced, the name MRSA has persisted and can be regarded as referring to resistance to virtually all β-lactam antibiotics. Susceptibility testing now typically uses oxacillin and/or cefoxitin. β-Lactams bind to the penicillin-binding proteins (PBP) essential for cell wall biosynthesis and inhibit peptidoglycan crosslink formation, leading to bacterial cell lysis. Resistance to β-lactams in MRSA is conferred by the acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene which encodes an altered PBP – PBP2a/PBP2′ – which has reduced affinity for β-lactam antibiotics. As a result, cell wall biosynthesis in MRSA strains continues even in the presence of otherwise inhibitory levels of β-lactam antibiotics. The detection and diagnosis of MRSA in the clinical microbiology setting is very important both for informing the appropriate treatment of individual patients and also for the surveillance of MRSA. The gold standard for confirmation of MRSA is regarded as the molecular detection of either *mecA*, typically by PCR, or of PBP2a/PBP2′, usually by antibody detection with commercially available slide agglutination assays. Crucial to the reliability of these assays is the fact that *mecA* and PBP2a/PBP2′ are both highly conserved among MRSA isolates.

Discovery of *mecC* MRSA: genome sequencing to identify a novel resistance gene

An epidemiological study of bovine mastitis [5] led to the isolation in 2007 of a *S. aureus* isolate, LGA251, from a bulk tank milk sample in southwest England which was phenotypically MRSA (i.e., resistant to oxacillin and cefoxitin). At that time this in itself was immediately significant because it represented the first detection of MRSA in the UK dairy herd. However, confirmatory tests for the *mecA*

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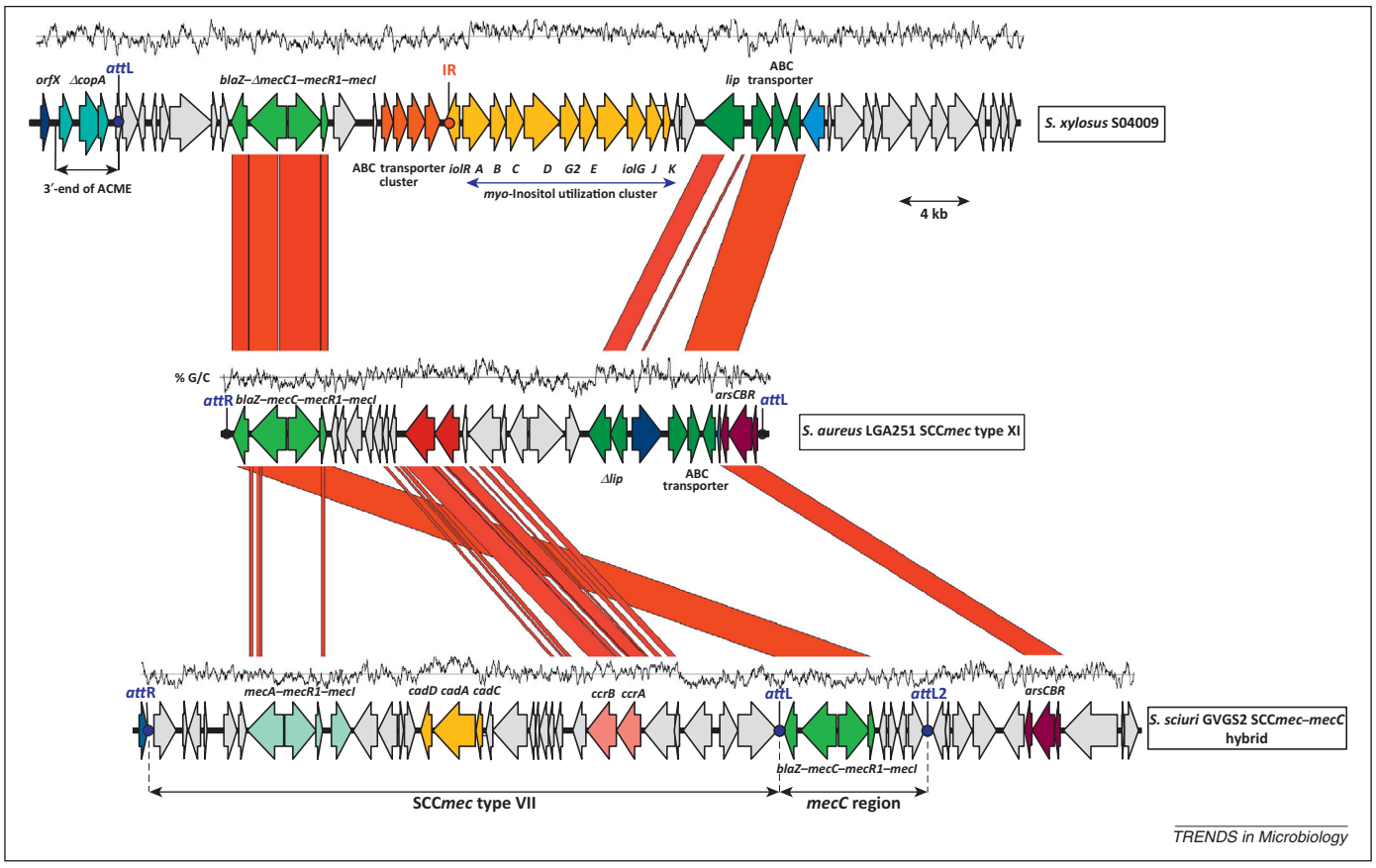


Figure 1. Comparison of the *mecC1* region in *Staphylococcus xylosus* strain S04009 [38] (EMBL accession number HE993884), SCC*mec* type XI in *Staphylococcus aureus* LGA251 [6] (EMBL FR821779), and a hybrid SCC*mec–mecC* in *S. sciuri* strain GVGS2 [39] (EMBL HG515014). Areas in red show regions conserved between the two sequences; homologous coding sequences are marked in the same colour. Blue and red dots indicate the SCC*mec* attachment sites (*att*L and *att*R) and inverted repeats (IR), respectively. The %G/C content of the region is shown above each genome schematic. Abbreviations: ABC, ATP-binding cassette; ACME, arginine catabolic mobile element; SCC*mec*, staphylococcal cassette chromosome.

gene and PBP2a/2′ were repeatedly negative [6]. Genome sequencing of LGA251 at the Wellcome Trust Sanger Institute revealed that the strain carried a novel *mecA* homologue, initially termed $mecA_{LGA251}$, which was only ~69% identical to conventional *mecA* at the DNA level, and the encoded PBP2a/2′ was ~63% identical at the amino acid level [6]. This explained the resistance of LGA251 and why it produced negative results by *mecA* PCR and PBP2a/2′ slide agglutination. A retrospective search of isolate collections in the UK and Denmark identified a further 65 isolates positive for $mecA_{LGA251}$ isolated not only from dairy cattle but also from humans, including the earliest known isolate, a Danish blood isolate from 1975 [6]. In consequence, although $mecA_{LGA251}$ MRSA has only recently been recognised, it may have been causing human infections for over 35 years. These $mecA_{LGA251}$ MRSA isolates belonged predominantly to CC130 and ST425 [6]. Similarly to conventional *mecA*, $mecA_{LGA251}$ is located within a SCC*mec* element inserted into the 3′ region of *orfX* (Figure 1). The LGA251 SCC*mec* was also novel; in other words, it had divergent *ccrA* and *ccrB* recombinases (belonging to the *ccrA1* and *ccrB3* groups and representing a novel combination of recombinase groups designated type 8 *ccr*), divergent *mecA* regulatory genes (*mecI*/*mecR*), and the absence of one of the three joining regions (J3) that are normally present [6]. The SCC*mec* sequence from LGA251 was submitted to the Working Group on the Classification of SCC and given the designation type XI SCC*mec* in November 2009. $mecA_{LGA251}$ was itself subsequently renamed *mecC* in 2012 [7]. *mecC* was chosen because an additional divergent homologue of *mecA*, distinct from $mecA_{LGA251}$, had already been described in *Macrococcus caseolyticus* [8] and was designated *mecB* [7]. Published at the same time as the UK and Danish report [6], work in the Republic of Ireland independently described *mecC* and type XI SCC*mec* in human MRSA strains isolated in 2010 and belonging to CC130 [9].

Functional characterisation of *mecC*-encoded PBP2a

The function of the *mecC*-encoded PBP2a/2′ and its role in β-lactam resistance was formally demonstrated by the work of Kim *et al*. which also highlighted noteworthy differences in the properties of the *mecA* and *mecC*-encoded proteins [10]. Although the detection of *mecC*-encoded PBP2a in LGA251 was problematic, most likely due to low expression levels resulting from *mecI*/*mecR*, inducible expression of *mecC* in a methicillin-sensitive *S. aureus* (MSSA) strain conferred high minimum inhibitory concentration (MIC) values against a range of β-lactams [10]. Recombinant $PBP2a_{mecC}$ protein was bound by β-lactams but showed higher affinity for oxacillin compared to cefoxitin, whereas $PBP2a_{mecA}$ showed less preference. The two proteins also displayed differences in their thermostabilty and temperature optima, with $PBP2a_{mecC}$ appearing to be

less stable at 37 °C than PBP2a$_{mecA}$. Interestingly, PBP2a-$_{mecC}$ did not require the presence of the native PBP2 to confer high-level oxacillin resistance. This is in contrast to PBP2a$_{mecA}$ for which high-level oxacillin resistance requires the presence of native PBP2 to provide transglycosylase activity lacking in PBP2a$_{mecA}$. Because PBP2a-$_{mecC}$ also appears to lack transglycosylase activity, high-level oxacillin resistance conferred by *mecC* is likely to involve collaboration between PBP2a$_{mecC}$ and one of the other monofunctional glycotransferases that are known to be induced in *S. aureus* when PBP2 is inhibited [10]. Although this characterisation confirms the function of *mecC*-encoded PBP2a as a transpeptidase, and its role in methicillin resistance, there are important differences in the behaviour of the proteins encoded by *mecC* and *mecA*. The structural and evolutionary bases for these distinctions are not yet clear.

The issue of *mecC* MRSA detection

Although there are obviously differences in biochemistry between *mecA* and *mecC*-encoded PBP2a, *mecC* nonetheless confers methicillin resistance, and such strains need to be identified correctly as MRSA in diagnostic laboratories. Where laboratories are performing antimicrobial susceptibility testing, *mecC* MRSA will likely be correctly identified as MRSA. Importantly, cefoxitin has been found to be more reliable than oxacillin in disc diffusion, broth microdilution, and agar dilution assays [11]. However, significant differences in the reliability of agars from different manufacturers have been described [11].

Similarly, *mecC* MRSA produce a distinctive antibiotic susceptibility profile compared to *mecA* MRSA when assayed using the automated Vitek 2 system from BioMérieux [12]. Where both oxacillin and cefoxitin are included, *mecA* MRSA, as might be expected, typically display resistance to both. By contrast, the majority of *mecC* MRSA show resistance to cefoxitin, and are therefore reported as MRSA, but however show susceptibility to oxacillin. Testing of a panel of 896 *S. aureus* isolates (comprising *mecA* MRSA, *mecC* MRSA, and *mec*-negative MSSA) found that this oxacillin-sensitive/cefoxitin-resistant profile had a sensitivity of 88.7% and a specificity of 99.5% for the identification of *mecC* MRSA isolates from MSSA and *mecA* MRSA [12]. This profile therefore provides a zero-cost screening method for identification of *mecC*-positive MRSA strains in the many clinical laboratories already using Vitek 2, although subsequent PCR would be needed to confirm *mecC* status. The performance of other automated systems for the detection of *mecC* MRSA resistance has not been fully tested and reported.

The differences in oxacillin and cefoxitin sensitivities displayed by *mecC* MRSA isolates are consistent with the findings of Kim *et al*. discussed above, demonstrating that the *mecC*-encoded PBP2a, unlike the *mecA*-encoded counterpart, has a higher relative affinity for oxacillin than for cefoxitin, leading to higher levels of resistance to cefoxitin than to oxacillin [10].

mecC MRSA appear to grow reliably on commercial chromogenic agar plates designed to identify MRSA, although there are indications that some MRSA agars may perform better than others for the recovery of *mecC* MRSA [13]. *mecC* MRSA typically have lower MICs to oxacillin and cefoxitin than their *mecA* counterparts, and this may affect their recovery on selective agars.

One major problem is where molecular detection of *mecA* is used to identify or confirm MRSA. Laboratories using this approach, most often PCR, will need to consider incorporating universal *mec* gene primers able to amplify both *mecA* and *mecC* or the addition of *mecC*-specific primers. This latter option has the benefit of differentiating *mecC* MRSA, thereby facilitating their surveillance and the isolation of strains for further characterisation. Various modified PCR assays have been developed to detect and/or differentiate *mecC* MRSA [14–16], and many commercial PCR-based assays are being, or have been, modified to include *mecC* detection [14,17]. Commercial slide agglutination assays for *mecA*-encoded PBP2a will also misidentify *mecC* MRSA as being methicillin-susceptible. These tests may be modified in due course to detect *mecC* MRSA, but currently the use of commercial slide agglutination assays alone will produce false-negative results for these strains. Strains found to be phenotypically resistant but *mecA* and/or PBP2a-negative are potentially *mecC* MRSA, and *mecC* PCR would be warranted to confirm this. *mec* gene-negative MRSA have also been reported [18].

In summary, *mecC* MRSA pose a potential diagnostic loophole which clinical microbiology laboratories should be aware of and which will require validation of testing approaches to ensure that *mecC* MRSA are correctly identified as MRSA. Statistically robust, formal studies are needed to validate the diverse MRSA susceptibility testing regimes for their correct identification of *mecC* MRSA as methicillin-resistant, even if only to confirm that current methods are sufficient.

Epidemiology of *mecC* MRSA in humans and animals

Following the original discoveries of *mecC* MRSA in the UK, Denmark, and the Republic of Ireland such strains, both human and animal origin, were rapidly identified in a further 10 Western European countries (Table 1). In many cases these reports represent small numbers of isolates identified by opportunistic sampling; for example, retrospective testing of previously identified atypical MRSA isolates. From these data it is unclear how common *mecC* MRSA truly are. In Denmark, however, where reporting of human MRSA is mandatory and extensive strain collections are maintained, the prevalence of *mecC* MRSA among all MRSA was found to be 1.9% in 2010, increasing to 2.8% in 2011 [19]. Further evidence supporting a recent increase is that very few Danish *S. aureus* isolates collected prior to 2003 were found to be *mecC* MRSA [19]. By comparison, large-scale collection and characterisation of human MRSA in Germany found only two *mecC* MRSA isolates among 3207 MRSA isolates (prevalence 0.06%), with no indication of a change in prevalence between 2004/05 and 2010/11 [20]. In the UK, a study in England during 2011–2012 surveyed 335 sequential MRSA isolates from individual patients collected from each of six clinical microbiological laboratories, and found a prevalence rate for *mecC* MRSA of 0.45% (nine *mecC* MRSA isolates from a total of 2010 MRSA isolates collected) [21]. The screening

Table 1. Distribution and characteristics of reported *mecC* MRSA

Country	Host species	Earliest reported isolate	*spa*-types	Multilocus sequence types (clonal complex)	Refs
UK	Human, dairy cattle, wild common seal, wild chaffinch, domestic dog	1993	t6300, t6292, t6220, t843, t6293, t1736, t1535, t7947, t7485, t7946, t7945, t6383, t742, t7734, t978, t6594, t7914, t9376, t6386, t9605, t8833, t11702, t11706, t9280	ST425 (CC425), ST130 (CC130), ST1245 (CC130), ST1526 (CC130), ST1944 (CC130), ST1764 (CC130), 1943 (CC1943/1946), ST1945 (CC130), ST1946 (CC1943/1946), ST2179 (CC599)	[6,15,21,26,33]
Denmark	Human, cattle, sheep	1975	t373, t528, t6220, t9397, t978, t2345, t3391, t8835, t9395, t843, t1535, t528, t1773, t1048, t3256, t1532, t1736, t3218, t3570, t5970, t9397, t5930 and t7603.	ST130 (CC130), ST1943 (CC130)	[6,33,42,43]
Republic of Ireland	Human	2010	t843 and t373	ST130 (CC130), ST1764 (CC130)	[9]
Germany	Human, wild hare, sheep, domestic dog, domestic cat, domestic guinea pig	2004	t843, t10513, t1736, 1773, t978, t7189, t1535, t10033, t10006, t1694, t278, t10009	ST130 (CC130), ST1945 (CC130), ST599 (CC599), ST2361 (CC1943/1946)	[13,20,37,44,45]
France	Human, dairy cattle	2007	t9280, t843	ST130 (CC130), ST1945 (CC130)	[31,46]
The Netherlands	Human	Not provided	Not provided	Not provided	[35]
Belgium	Wild brown rat, farmed rabbit, dairy cattle, beef cattle	1995	t208, t742, t9925, t1736	ST2273 (CC49), ST425 (CC425), ST2508 (CC599), ST130 (CC130)	[33,47]
Sweden	Dairy cattle, wild hedgehog	2003	t524, t9111	ST130 (CC130), ST425 (CC425)	[36,48]
Norway	Domestic cat	2012	t6902	ST2497 (CC1943/1946)	[32]
Austria	Wild European otter and wild European hedgehog	Winter 2012/13	t4335 and t3256	ST2620 (CC130), ST130 (CC130)	[37]
Spain	Human	2008	t843 and t6220	ST130 (CC130), ST1945 (CC130)	[30,49]
Switzerland	Human	2011	t11150	ST130 (CC130)	[22]
Finland	Dairy cattle	2006	t3256	ST130 (CC130)	[50]

of 565 *S. aureus* isolates collected between 2005 and 2011 in western Switzerland did not identify any *mecC* MRSA isolates, suggesting that these are also rare in that country [22]. *mecC* MRSA has yet to be reported from outside Western Europe, and a small survey of US service personnel injured during deployment in Iraq and Afghanistan and transitioned through Germany *en route* to the USA found no *mecC* MRSA among 102 MRSA isolates [23]. *mecC* MRSA currently appears to be uncommon in humans, but there are interesting geographical differences in prevalence; the recent increase in Denmark highlights the need to monitor *mecC* MRSA.

Although a number of multilocus sequence types have been found among *mecC* MRSA isolates, two major lineages are responsible for the vast majority of isolates to date: CC130, which seems to predominate, and ST425. Among these lineages a large number of *spa*-types are represented (Table 1), with t843, associated with CC130, being the most common. *mecC*-negative ST425 have also been reported [24], as have CC130 MSSA, although the *mec* gene status of the latter was not confirmed [25].

mecC MRSA have been found in a wide range of other host species encompassing livestock, wildlife, and companion animals from many European countries (Table 1). As with human isolates, these isolates predominantly belong to CC130 and to a lesser degree ST425. These lineages therefore appear to have a very broad host tropism. There are few data on the prevalence among animals, although a British study of bovine bulk tank milk found that 2.67% of dairy farms in England were positive for *mecC* MRSA but, interestingly, no positive farms were found in Scotland during the same survey [26]. Assessing the prevalence of *mecC* MRSA among different livestock species, understanding their role in veterinary disease, and the risk of zoonotic transmission are important topics for future research.

Zoonotic potential of *mecC* MRSA

Both CC130 [27] and ST425 [6] have previously been regarded as animal-adapted lineages of *S. aureus*, suggesting that *mecC* MRSA arose in animals, possibly ruminants, and subsequently spread to humans [6]. Although the origins of *mecC* MRSA are not yet clear there is good evidence that contact with animals poses a zoonotic risk and that *mecC* MRSA can be transmitted between species – and therefore could be regarded as a LA-MRSA. For instance, most isolates in Denmark come from rural areas [19], and epidemiological follow-up of 22 patients found known animal contact in four cases [19]. Genome sequencing of *mecC* MRSA isolates from two of these cases provided compelling evidence of cross-species transmission with human and individual animal (cow and sheep) isolates being separated by only a few single-nucleotide polymorphisms across the entire core genome [28]. A survey of delegates at British Cattle Veterinary Association Congress in 2011 failed to find any *mecC* MRSA, providing evidence that the prevalence in this population is below 1% [29]. However, many cases of *mecC* MRSA do not have apparent animal contact, and household transmission between people has been demonstrated [30].

mecC MRSA and disease in humans and animals

mecC MRSA have been isolated from carriage and a range of infections in humans (see Table 1 for list of references). These are predominantly skin and soft-tissue infections but include severe bone infections [31], nosocomial pneumonia [13] and fatal bacteraemia [30]. *mecC* MRSA can also cause disease in veterinary species; for example, mastitis in dairy cattle has been noted in several countries, and other examples include chronic conjunctivitis in a domestic cat [32] and a rabbit isolate of *mecC* MRSA from Belgium belonging to a highly virulent clone among farmed rabbits [33,34]. It appears that, similarly to conventional MRSA lineages, *mecC* MRSA strains are highly-versatile pathogens able to cause a wide range of infections in a range of host species, including severe and fatal infections. In agreement with these epidemiological observations, microarray analysis and genome sequencing reveal that *mecC* MRSA isolates encode several known or putative *S. aureus* virulence factors, including several adhesins, superantigens, and toxins [13,28,35]. In addition, a novel allele of *etd* (encoding exfoliative toxin D) with only 59% identify to the previously described *etd* gene was identified in CC130 isolates, and was putatively named *etd2* [28,36]. However, where tested, *mecC* MRSA strains have been negative for Panton–Valentine leukocidin [15,19], a prominent virulence factor among CA-MRSA, and they have been negative for the human immune evasion genes *sak*, *chp*, and *scn* [13,35], consistent with a possible origin for these strains in an animal reservoir.

Resistance to non-β-lactam antibiotics is currently uncommon among *mecC* MRSA isolates, and MICs for oxacillin and cefoxitin are generally low compared to those that can be seen among *mecA* MRSA. It will be of interest and of potential importance to monitor if these features change in the future.

mecC in other species of staphylococci

The origins of *mecC* MRSA and SCC*mec* type XI are unclear, but *mecC* has also been detected by PCR in *Staphylococcus stepanovicii* from a wild European lynx in Austria [37], and a homologue, *mecC1*, located within a SCC*mec* IX-like element has been described using genome sequencing of *Staphylococcus xylosus* isolated from bovine milk in France [38] (Figure 1). This latter gene has 93.5% sequence identity to *mecC* in MRSA and is therefore classed as an allotype of *mecC* (≥70% but <95% nucleotide sequence identify) [7,38]. Finally, *mecC* has been found in *Staphylcoccus scirui* within a novel hybrid SCC*mec*–*mecC* element in isolates from caesarean incision wounds in Belgian Blue cattle [39] (Figure 1). As suggested for *mecA* [40,41], it is possible that *mecC* has its origin among coagulase-negative staphylococci; further investigations, including whole-genome sequencing of *mecC* staphylococci, may offer clues to the origin and evolution of this resistance determinant. These data also mean that clinical microbiology laboratories should be aware not only of *mecC* MRSA but also of the possible occurrence of *mecC* in other pathogenic species of methicillin-resistant staphylococci.

Box 1. Outstanding questions

- How widespread and common are *mecC* MRSA strains? To date they have only been reported in Western Europe, but are they present elsewhere, remaining undetected, or will they spread elsewhere?
- What are the cause(s) of the recent increase in prevalence reported from Denmark; will this continue and will this be seen elsewhere?
- How common are *mecC* MRSA strains among animal populations (wild, companion, and livestock), and how important are they for animal health or as a zoonotic reservoir for human MRSA infections?
- How and where did *mecC* and SCC*mec* type XI originate, and when and how often have they been acquired by *Staphylococcus aureus*? Why is CC130, and to a lesser degree ST425, so dominant among *mecC* MRSA isolates? Will it spread to other lineages, and will current *mecC* MRSA lineages acquire additional resistance determinants?
- What is the basis for the apparent broad host specificity shown by the *mecC* MRSA lineages CC130 and ST425?
- What is the structural and evolutionary basis for the functional differences between *mecA* and *mecC*-encoded PBP2a/2′?
- What are the best methods (e.g., chromogenic plates or antimicrobial susceptibility testing approaches) to detect *mecC* MRSA? Do diagnostic work-flows need to be altered to improve *mecC* MRSA detection?
- What is the distribution and prevalence of *mecC* among other staphylococci, and do these contribute to disease in animals and humans? Did they play a role in the origin of *mecC* and its dissemination to *S. aureus*?

Concluding remarks

mecC MRSA represent a recently recognised form of MRSA, encoding a divergent *mec* gene, which can colonise and cause disease in humans and a wide range of other host species. Although *mecC* MRSA are currently rare, and have only been reported in Europe to date, they present a potential diagnostic problem where there is reliance on *mecA* or PBP2a/2′ detection for MRSA diagnosis, and their emergence raises a several questions for future research (Box 1).

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