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Source / Izvornik: **Medical Hypotheses, 2019, 126, 38 - 41**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.1016/j.mehy.2019.03.011

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:168186

Original Paper

A ‘pathogenic needle’ in a ‘commensal haystack’: genetic virulence signatures of *Corynebacterium glucuronolyticum* that may drive its infectious propensity for the male urogenital system

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Abstract

The predominance of the genus *Corynebacterium* in the healthy male urogenital system contributes to the resident microbiome of not only the distal urethra, but potentially the proximal urethra and urinary bladder as well. However, for certain species in this genus, pathogenic potential was described, and the salient representative is *Corynebacterium glucuronolyticum* (*C. glucuronolyticum*) implicated in cases of urethritis and prostatitis in men. Nonetheless, some still question whether *C. glucuronolyticum* can actually be considered pathogenic or rather just a commensal species fortuitously isolated in patients with urogenital symptoms and/or syndromes. Although pathogen/commensal dichotomy is not always clear-cut, we hypothesize that specific genetic markers may expose *C. glucuronolyticum* as a convincingly pathogenic *Corynebacterium*. More specifically, characteristic pathogenic gene constellation inherent to this species (most notably the presence of specific sortase/SpaA-type pili gene clusters, but also the augmentative role of type VII secretion system) may significantly facilitate host tissue adhesion, with subsequent suppression/evasion of the immune response and acquisition of vitally important nutrients. Consequently, these genetic markers differentiate *C. glucuronolyticum* from its commensal counterparts, and give this species a pathogenic facet, which can be even further influenced by the Allee effect. In this paper we also propose a specific methodological approach on how to analyze *C. glucuronolyticum* epithelial colonization capacity and explore inceptive host cell-pathogen interactions that manipulate host environment and immune responses. This entails moving from approaches based primarily on overall homology of primary sequences towards specific structure-function studies to precisely evaluate all stakeholders involved in pili assemblage, cell adhesion and the expression of other virulence traits. In the era of high precision medicine, the hypothesized roles of *C. glucuronolyticum* adhesion systems in both virulence and nutrient acquisition may also reveal promising targets for future drug developments.

Introduction

Corynebacteria, which is a diverse group of pleomorphic and asporogenous Gram-positive bacilli from the class *Actinobacteria*, are widely regarded as commensal flora of the urogenital tract in men – akin to *Lactobacillales* species in women [1]. Some researchers even pointed out that such predominance of *Corynebacterium* genus in healthy males contributes to the healthy microbiome of not only the distal urethra, but potentially the proximal urethra and urinary bladder as well [2]. In that regard, this group of bacteria acts as markers and harbingers of adequate urogenital health in men.

However, there is a cornucopia of various species in this genus, which means that the automatic clustering of all corynebacterial isolates from the urogenital tract as commensal flora may result in under recognition of some possibly pathogenic ones. And indeed, in recent years there were various reports in medical literature of several *Corynebacterium* species that were implicated in infections of the male urinary and/or genital tract, such as *Corynebacterium urealyticum*, *Corynebacterium striatum*, *Corynebacterium propinquum* and *Corynebacterium aurimucosum* [3-6]

The salient representative of this potentially pathogenic cluster of species is *Corynebacterium glucuronolyticum* (previously known as *Corynebacterium seminale*), after various researcher groups repeatedly addressed its clinical significance in men without any known immunodeficient states [7-13]. Although initially described as a cause of acute urethritis [8-10] and identified in certain cases of prostatitis [11], it was shown that this species may have certain influence on semen parameters as well [12]. The first classification of genitourinary syndromes caused by *C. glucuronolyticum* was also recently published in the medical literature (Mestrovic's classification) [13].

Nonetheless, some still question whether *C. glucuronolyticum* can actually be considered pathogenic or rather just a commensal species fortuitously isolated in patients with urogenital symptoms and/or syndromes. Of course, pathogen/commensal dichotomy is not always clear-cut, and host factors can influence the often delicate balance between these two states. The question is whether there are certain genetic, more objective markers that may point towards *C. glucuronolyticum* as a convincingly pathogenic corynebacterium.

Hypothesis

We hypothesize that characteristic pathogenic gene constellation inherent to *C. glucuronolyticum* – most notably the presence of specific sortase/SpaA-type pili gene clusters – significantly facilitates host tissue adhesion, with subsequent suppression/evasion of the immune response and acquisition of vitally important nutrients. These genetic markers may, therefore, differentiate *C. glucuronolyticum* from its commensal counterparts.

Evaluation of the hypothesis and the rationale behind it

In corynebacteria, cell wall proteins represent key determinants of host adhesion and colonization, while various sortase enzymes likely enhance their adhesive capacity [14]. These enzymes are included in the formation of pili that are covalently attached to bacterial cell wall and serve as major adhesins – not only in pathogenic corynebacteria, but in other bacterial pathogens as well [15,16]. More specifically, the assembly of corynebacterial pili employs a two-step mechanism, where pilins are covalently polymerized by a pilin-specific sortase enzyme, and the generated pilus polymer is then anchored to the cell wall peptidoglycan by a non-polymerizing or housekeeping sortase [14].

All known sortases are cysteine transpeptidases that join proteins to the peptidoglycan of the cell wall, or bind proteins to construct pili [17]. Accessory sortase genes (*srtA-E*) are known to encode enzymes that facilitate covalent linkage of extracellular LPxTG proteins and thus play an important role in infection pathogenesis. These are pervasive only in *Corynebacterium diphtheriae* (*C. diphtheriae*), *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis* [18].

Sortase genes *srtA-E* have to be differentiated from the housekeeping sortase (*srtF*), since the latter has a role in the cell in anchoring of pili, but does not polymerize pilins [18,19]. These *srtA-E* genes are encoded within three well-defined pilus gene loci responsible for encoding SpaA-type pili (genes *spaA*, *srtA*, *spaB*, *spaC*), SpaD-type pili (genes *srtB*, *spaD*, *srtC*, *spaE*, *spaF*), as well as SpaH-type pili (genes *spaG*, *spaH*, *srtD*, *srtE*, *spaI*), respectively [18].

Rogers *et al.* described pilus gene clusters in pathogenic *Corynebacterium* species and showed how *C. glucuronolyticum* is characterized by SpaA-type pili bundle [14]. There is also a presence of housekeeping sortase, *srtF*, which is located elsewhere on the chromosome [14]. The prototype SpaA pilus is composed of SpaA forming the pilus shaft, with two minor pilins: SpaB (located at

the base) and SpaC (located at the tip) [14]. These minor pilins (SpaB and SpaC) were described as being pivotal for binding to human cells by previous research [20]. In other words, they act as major adhesins in different cells.

However, significant variations in the presence or absence of different pilus gene clusters have previously been described [14]. From the work of Heydari *et al.* on *Corynebacterium* genomic database and their useful pathogenomic profiling tool for comparative virulence gene analysis, it is strikingly evident that many pathogenic strains of corynebacteria are characterized by only partial pilus loci [18]. For example, *srtD* and *srtE* genes can be seen in five *C. diphtheriae* strains exhibiting the lack of *spaG*, *spaH* and *spaI* [18].

Likewise, in the aforementioned study by Heydari *et al.* [18], *C. glucuronolyticum* is characterized with the presence of *srtA* and *srtC* genes, which are absent in a wide array of other coryneform bacteria (more specifically, many do not harbor any *srt* genes). We hypothesize that these genes may be crucial virulence factors found in *C. glucuronolyticum*, since they act to display surface proteins – mediating in turn bacterial adhesion to host tissues, and potentially invasion, suppression/evasion of the immune response, as well as the acquisition of vitally important nutrients. This particular subset may represent an important evolutionary advantage when compared to many other *Corynebacterium* species.

Ott and coworkers already demonstrated that different isolates of *C. diphtheriae* are characterized by a different pili repertoire whose polymerization depends on sortases, which is then marked by distinct biophysical properties [21]. We believe that even an incomplete repertoire of sortases and pilus gene clusters found in *C. glucuronolyticum* may be sufficient to exert pathogenic properties in the urogenital system by adhering to and damaging cells present in that particular anatomical region.

Our hypothesis is also supported by a seminal paper by Sangal *et al.* where 20 *C. diphtheriae* genome sequences where compared and analyzed by employing different bioinformatic approaches [22]. The authors clearly demonstrated a correlation between the structure/organization of *spa* gene clusters and the potential of different non-toxigenic *C. diphtheriae* strains for host cell adherence and invasion. More specifically, the paper highlights the variations in the secreted proteome (particularly mentioning proteins implicated in pili synthesis and those with LPxTG motifs) and its potential association with the degree of pathogenesis [22]. As highly specific sortase gene clusters in *C. glucuronolyticum* seem even more stable and pervasive across the species, we believe this further corroborates our hypothesis.

In addition to sortases, a single type VII secretion system can be observed in the *C. glucuronolyticum* core proteome, congruous with previous studies [23]. The core components of this system are EccB, EccC, EccD and a mycP protease, type VII-secretion associated protein from the Rv3446c family and WXG100 family type VII-secretion target [24]. The latter represent the principal substrate for this specialized system known to influence virulence in related bacteria [23,25].

We further hypothesize that type VII secretion system is important for both colonization and persistence of *C. glucuronolyticum* in the male urogenital system, augmenting primary virulence traits provided by the aforementioned sortases and pilus gene cluster. Studies on *Staphylococcus aureus* in mouse models have found that the disruption of similar systems (or substrate deletion) may result in substantially reduced bacterial numbers and, in turn, diminished bacterial pathogenicity [25,26]. Although type VII secretion system is present in other corynebacteria that are considered commensal, a hypothesis is that a compounding effect, which supplements the activity of sortases, is what gives this species the evolutionary edge and a pathogenic facet.

Other virulence factors are also annotated in the *C. glucuronolyticum* genome, most notably different serine-proteases (rhomboid family intramembrane serine protease, MarP family serine protease) and metallo-proteases (RIP metalloprotease, zinc-dependent metalloprotease), as well as iron ABC transporter systems [24,27,28]. However, since these genetic traits are pervasive in other *Corynebacterium* species as well, we hypothesize that they have an auxiliary role in the disease pathogenesis – *i.e.* only after primary pathogenetic event takes place due to sortase/SpaA-type pili gene cluster products and their interaction with urothelial cells. There are also several 'hypothetical proteins' highlighted in *C. glucuronolyticum* genome which may also participate in the disease pathogenesis, but further genomic and functional insights are needed to elucidate their exact role [24,27,28].

The propensity to attach to surfaces of host cells is obviously a key initial step in colonization, since this can hamper host clearance mechanisms through shear stress [29]; we see this in other frank urogenital pathogens as well (such as *Neisseria gonorrhoeae*) [30]. Nonetheless, attachment by itself is not sufficient to establish and maintain an infectious process. It is known that bacteria have evolved mechanisms of host environment and immune response manipulation to aid their survival and spread by alteration of host cell signaling [29]. With such genetic pedigree, *C. glucuronolyticum* may also be in a pole position to directly manipulate host cell signaling through the process of adhesion in urogenital niche environment, akin to some other bacterial species [29,30].

Furthermore, the Allee effect could also play a role, which is our additional hypothesis that also warrants further research. More specifically, the Allee effect describes the potential of many microorganisms to exhibit advantageous and pathogenic traits when present in high population densities [31,32]. For example, it was shown that in pathogenic *Staphylococcus aureus* a minimum population density is most often needed to commence expression of its virulence factors [33]. If this effect exists in *C. glucuronolyticum* as well, it may explain why there are certain adverse effects on

sperm parameters when this organism is present as a monoisolate in high numbers [12], and reflects different selective pressures that affect optimal population density in the urogenital environment.

No plasmids have been described in *C. glucuronolyticum*, and the species in question lacks many genes present in its more pathogenic counterparts [18], which means its pathogenicity may mostly depend on described adhesion and invasion properties conferred by joint action of sortases and type VII secretion system. Furthermore, previous studies have shown that, when compared to some other coryneform species, *C. glucuronolyticum* does not show the propensity to form biofilms in the prostate gland [34]. This may be one of the reasons why urethritis syndrome is the most common clinical manifestation observed in relation with this bacterial agent.

Towards experimental proof of the hypothesis

Compared to the body of literature on a large number of bacterial pathogens, there are some serious gaps in our knowledge on corynebacterial adherence. The first report describing corynebacterial pili and their role in mediating host cell interactions was done by using *Corynebacterium renale* as the test species, which successfully agglutinated trypsinized sheep red blood cells [35]. In 2007, Mandlik *et al.* singled out minor pilins SpaB and SpaC in *C. diphtheriae* as specific adhesins that are responsible for efficient adherence to host pharyngeal cells [20]. Wild-type *C. diphtheriae* strains were shown to bind successfully to lung epithelial, pharyngeal and laryngeal cells, but various mutants without certain pilin subunits lost that ability [20].

C. glucuronolyticum belongs to the group of sequenced *Corynebacterium* species with one or more pilus gene clusters, as well as specific types of pilins and pilus-specific sortases present in its genome [14,18]. Since the composition of these genes shows a characteristic signature, in order to prove this hypothesis it will be essential to establish whether specific gene clusters and combinations mentioned in this paper encode pathogenic pilus structures similar to those in *C. diphtheriae*, and whether these are also instrumental for its interaction with specific urethral and uroepithelial cells.

Therefore, in order to test our hypothesis, prolonged *in vitro* bacteria-epithelium infection experiments should be simulated with the use of flow chamber-based infection models [36]. Such models are able to facilitate the investigation of adherence and bacterial interactions with epithelial cells while subjected to physiological liquid shear [37-40]. Furthermore, adhesion stimuli could be induced in *C. glucuronolyticum* and the impact of the physiological microenvironment on its pathogenesis could be thoroughly researched with this approach.

In such a model, wild-type *C. glucuronolyticum* would be compared with mutants that lack certain combinations of sortase and SpaA-type pili gene clusters. This would show whether there is a reduction in adherence to uroepithelial and other types of cells when mutants are compared to wild-type strains. Quantification of bacteria colonizing flow chamber-cultured uroepithelial and other cell layers would be based on fluorescence signals emitted by adherent bacteria that constitutively express green fluorescent protein (GFP), an approach recently described in the literature [36]. In addition, this model could be broadened by interrogating the ability of latex beads coated with only certain pilin subunits specific for *C. glucuronolyticum* to adhere to *in vitro* cells.

Our proposed methodology makes detailed analysis of *C. glucuronolyticum* epithelial colonization capacity feasible and opens doors for important investigations of inceptive host cell-pathogen interactions that manipulate host environment and immune responses. Future models that would allow controlled growth of cell layers with differentiated epithelium (*e.g.* 3D cultures or cell exfoliation models), as well as stimulated mucoid layers, may mimic inflammation responses and infection with immense precision – especially when specific immune factors are added [36]. This could then be used to study suppression/evasion of the host immune response.

On a broader level, to test this hypothesis it is important to move from approaches that are based primarily on overall homology of primary sequences by pinpointing specific enzyme features that provide function and virulence to *C. glucuronolyticum*. In other words, structure-function studies would be needed to identify characteristic enzyme determinants that guide substrate specificity. This could elucidate how *C. glucuronolyticum* sortases selectively steer different proteins to the cell surface by discerning their unique sorting signals, and unveil how this species can attach proteins to its cell wall or assemble pili.

Many questions are still looming with regards to the function and structure of type VII secretion systems and their individual substrates, which have a prominent role in pathogenic mycobacteria [24], and could augment adherence in this species as well. Also, the potential presence of the Allee effect in *C. glucuronolyticum* could be tested with development assays, culture biovolume density determination, and with the use of the aforementioned cell layers – appraising the species' colonizing abilities and other potentially pathogenic traits in the presence of a high burden of microorganisms [29].

Conclusions

According to the published literature thus far, *C. glucuronolyticum* is increasingly being recognized as a potential pathogen, and not merely a commensal corynebacterial species. Its unique genetic signature could well mean that this species demonstrates colonizing abilities comparable to some other, well-established urogenital pathogens. However, to firmly corroborate its pathogenic potential, future approaches will have to include a wide array of structure-function studies to precisely evaluate all stakeholders involved in pili assemblage, cell adhesion and the expression of other virulence traits.

In the meantime, the improved availability and continuous updates of new genome sequences in the corynebacteria database and the addition of advanced tools will be used to further analyze the genomic data at hand. In the era of high precision medicine, the hypothesized roles of *C. glucuronolyticum* adhesion systems in both virulence and nutrient acquisition may also reveal promising targets for future drug developments.

Conflict of interest statement

All authors declare that there is no conflict of interest in this study.

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