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Identification and function of bacterial small RNAs in human circulation

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Abstract: Small RNAs (sRNAs) are a class of post-transcriptional regulators in bacteria and eukaryotes. Recent advancements in small RNA sequencing have unveiled the presence of bacterial sRNAs in human body fluids, indicating that these sRNAs might be transmitted into the host organism via extracellular vesicles (EVs). This discovery underscores the potential of bacterial sRNAs as novel communicators in host-microbe interactions and as biomarkers for disease diagnosis and progression. Additionally, the review delves into the role of bacterial sRNAs within EVs, highlighting their significance in mediating bacterial virulence and modulating host immune responses. The emerging application of sRNAs in the early detection of bacteremia presents a novel approach to diagnosing and managing bacterial infections, showing the potential of sRNAs as a tool in clinical settings.

Keywords: bacterial small RNA (sRNA); extracellular vesicle (EV); human circulation

1. Introduction

Small RNAs (sRNAs) are a diverse group of non-coding RNA molecules, that play crucial roles in regulating gene expression in both bacteria and mammals [1]. A common mechanism through which both bacterial and mammalian sRNAs act is post-transcriptional gene silencing, either by targeting mRNAs for degradation or by inhibiting their translation [2]. Bacterial sRNAs typically 50–500 nucleotides in length, are directly transcribed from intergenic regions, untranslated regions (UTRs), or antisense to coding sequences [3–5]. Since the first bacterial sRNA 6S RNA was discovered in *Escherichia coli* in 1984 [6], a large number of sRNAs (especially in *Escherichia coli*) have been identified and characterized [7,8]. According to action mechanism, bacterial sRNAs can be divided into cis-encoded antisense sRNAs, which can achieve fully complementary binding to their targets, and trans-encoded antisense sRNAs, which are only partially complementary to their targets, with binding facilitated by the host factor for RNA phage Qβ replicase (Hfq) [9,10]. Bacterial sRNAs are involved in multiple aspects of cell physiology, including the utilization of carbon sources, amino acids and metal ions, cellular cleavage, quorum sensing, bacterial biofilm formation, and virulence gene expression, and is crucial for the rapid response of stress conditions [11–13]. Nevertheless, there are certain differences between mammalian sRNAs and bacterial sRNAs. Mammalian sRNAs, with a shorter length (~ 18 to 30 nucleotides) [1], primarily including microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) [14], are processed from longer precursors by enzymes such as Drosha, Dicer, and PIWI [15,16]. Bacterial sRNAs exhibit a broad range of functions, which are specific to the needs of single-celled organisms. Mammalian sRNAs, on the other hand, are more involved in developmental processes, cellular differentiation, and the maintenance of genomic stability [17].

Recent advancements in molecular biology, particularly through the application of sRNA sequencing techniques, have led to the identification of bacterial sRNAs within human body fluids, bovine milk and mouse plasma [18]. This discovery has prompted further investigation into the potential roles of bacterial sRNAs in facilitating inter-organism communication [19]. One hypothesized mechanism for this communication is through extracellular vesicles (EVs) [20–22], which may serve as vehicles for the transfer of sRNAs between different biological entities [23]. In this review, we consolidate the current understanding of the identification and discovery of bacterial sRNAs within the human, and propose potential functions and roles they may serve in human physiological contexts.

2. Microbial sRNAs in extracellular vesicles

Bacterial EVs are membranous structures actively secreted by bacteria, with sizes ranging from 20 to 400 nanometers. These vesicles are classified based on their origin within the bacterial cell structure. They are known as outer membrane vesicles (OMVs) when derived from Gram-negative bacteria [24] and as cytoplasmic membrane vesicles (CMVs) when originating from Gram-positive bacteria [25]. The established names are somewhat deceptive, given our current understanding that vesicle variations can encompass elements from the inner membrane, periplasm, or cytoplasm [26]. The identification of bacterial EVs in 1967 represented an important advance in the field of microbiology. Observed initially in *Vibrio cholerae*, these EVs were captured as spherical membrane-bound structures through electron microscopy, a method that allowed scientists to visually confirm their existence directly from the bacterial outer membrane [27]. Initially, these vesicular entities were regarded as mere byproducts of cellular activity, lacking substantial functional relevance, and consequently, were largely ignored in the study of bacterial physiology and pathogenesis. As research methodologies improved and understanding deepened, perceptions of bacterial EVs have changed. It is established that these entities are not merely cellular debris; instead, they play essential roles in various biological processes, including virulence, horizontal gene transfer, export of cellular metabolites, phage infection, and cell-to-cell communication [28,29].

The advent of omics technologies has played a crucial role in revealing the diverse and abundant contents within these vesicles. These technologies have shown that EVs from both gram-positive and gram-negative bacteria contain a wide range of nucleic acids, proteins, lipids, and metabolites, which are enveloped within a protective lipid bilayer [30,31]. This bilayer is crucial as it protects intra-vesicular contents like RNA from degradation by external RNases and proteins from extracellular proteases, thereby preserving their functional integrity. Detailed characterization studies have further illuminated the specific roles and components of EVs in various bacteria. For instance, research on *Escherichia coli* by Ghosal *et al.* highlighted that extracellular RNA within EVs, particularly those ranging from 15 to 40 nucleotides, originated from distinct intracellular RNAs, suggesting a selective mechanism of RNA packaging and export [21]. Similarly, studies on periodontal pathogens such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Treponema denticola* have characterized msRNAs (miRNA-sized sRNAs) which modulated the host immune system via bacterial OMVs [32]. *Pseudomonas aeruginosa*, known for its high drug resistance, also utilized EVs to transfer specific sRNAs (sRNA52320) to host cells, potentially facilitating bacterial survival and proliferation [20]. Research into the identification of sRNAs in EVs has not been limited to bacteria alone but has extended to other organisms such as fungi and protozoa. Studies involving fungi like *Cryptococcus neoformans* and *Candida albicans* [33], and protozoa such as *Trypanosoma cruzi* [34] have demonstrated that EVs are a universal tool for RNA export , with potential implications in infection and immunity across a broad spectrum of life forms.

All of these studies emphasize the importance of sRNAs from microbial EVs as crucial communication molecules that are able to mediate host-microbe interactions. However, as of now, the mechanisms responsible for the sorting of small RNA (sRNA) into EVs, cellular targets, as well as involvement in biological regulation are largely unknown, which deserve more attention in future studies.

3. Discovery of bacterial sRNAs in the human circulation

Recent research has focused on identifying extracellular sRNAs in human circulation, revealing that a significant portion of these molecules originates from exogenous species [18,35,36]. Notably, these circulating RNAs are derived not only from dietary plant miRNAs but are predominantly bacterial or fungal in origin [37]. Despite the abundance of such findings, studies targeting bacterial sRNAs in human circulation remain scarce, offering only preliminary insights. In a seminal 2012 study, Wang *et al.* employed Next Generation Sequencing technology on nine plasma samples to precisely map the spectra of these vital molecules. The methodology excluded miRNA and utilized three levels of error tolerance—zero, one, and two mismatches—for endogenous sequence mapping. Unmapped sequences were further analyzed by comparing them against the known human microbiome, miRNA sequences from other species, and NCBI's non-redundant nucleic acid sequence collection. This comprehensive approach revealed a diverse array of RNA molecules from bacteria, fungi, and other species, suggesting their potential roles as signaling molecules or indicators of human health [18]. Similarly, Beatty *et al.* documented sequences of microbial origin in sRNA sequencing data from blood samples, with the majority of bacterial reads associated with the phylum *Proteobacteria*. In five out of six individuals analyzed, over 90% of the prevalent fungal reads were traced back to the phylum *Ascomycota*, particularly the order *Hypocreales*. Many sequences were also identified as originating from plants, likely reflecting dietary intake [36]. Moreover, Semenov *et al.* conducted extensively parallel sequencing of RNA from human blood plasma, detecting sRNAs that correlated with bacterial non-coding RNAs, specifically from the genera *Escherichia* and *Acinetobacter*, among other microorganisms [38]. The studies mentioned above prompt researchers to explore the potential biological applications of bacterial sRNAs in human circulation, which could lead to novel insights into their roles in human health and disease.

4. The function of bacterial sRNAs in the human circulation

4.1. Bacterial sRNAs in disease diagnosis

Indeed, it is widely acknowledged that extracellular RNAs (especially miRNAs) could be used as effective biomarkers for disease states and progression. In recent years, a rapidly expanding literature has highlighted the connection between circulating miRNA levels and various human diseases and pathologies [39–42]. Although the use of miRNA in clinical practice currently lacks standardized protocols, miRNAs are considered promising biomarker candidates for several reasons. Their stability in bodily fluids, ease of extraction from patients, and the potential for sensitive and easy measurement due to their amplifiability are significant advantages [43]. Similarly, bacterial sRNA serve not only as indicators of bacterial growth, adaptability, and metabolic states but also exhibit the potential to reflect variations in different physiological conditions and disease contexts. Consequently, they can be employed for the indication or diagnosis of specific diseases, infections, or physiological states. For instance, in tuberculosis, certain *Mycobacterium tuberculosis*-encoded sRNAs have been detected in patient plasma, suggesting that cell-free bacteria-specific sRNAs can be released into the circulation from infected tissues [44,45]. This finding opens the possibility of using bacterial sRNAs as biomarkers for early-stage diagnosis of such infections. In a quite recent study, Ormseth *et al.* performed sRNA sequencing on plasma from rheumatoid arthritis (RA) patients and matched controls. In the plasma of RA patients, a notable reduction was observed in the abundance of 10 microbial species as compared to the control group. Conversely, three sRNAs originating from microbial transfer RNAs exhibited elevated levels in RA patients when contrasted with control subjects, and these increases displayed an inverse correlation with disease activity. Additionally, a higher proportion of plasma microbial sRNAs was associated with lower RA disease activity in RA patients [46]. It is worth noting that the connection between bacterial host diseases has just begun to be explored and the potential of bacterial sRNA as biomarkers warrants further exploration and validation.

4.2. Bacterial sRNAs in pathophysiological conditions and in immune response

The significance of sRNAs in mediating interactions between the physiological microbiota and the host organism, particularly within the context of immune system functioning, cannot be overstated. sRNAs contribute to the maintenance of a delicate equilibrium between host defence mechanisms and microbial symbiosis, playing pivotal roles in immune modulation and homeostasis [47]. These molecules facilitate communication between microbiota and host cells, influencing immune cell differentiation, activation, and response [48]. For example, microbial-derived sRNAs can modulate host gene expression, impacting pathways involved in inflammation and immune tolerance [49]. Koeppen *et al.* initially characterized sRNAs from *Pseudomonas aeruginosa* in OMVs, showing that sRNA52320 transferred to host cells diminished both lipopolysaccharide(LPS) and OMV-induced IL-8 secretion in human airway epithelial cells and reduced the secretion of the KC cytokine and neutrophil infiltration in mouse lung. This suggested that the inhibition of innate immune responses occurs through the regulation of the mitogen-activated protein kinase (MAPK) pathways, which were critical for IL-8 synthesis [20]. Similar to the findings on *Pseudomonas aeruginosa*, Zhang *et al.* indicated that sRNAs in *Helicobacter pylori* were crucial in modulating the host immune response, with sRNAs sR-2509025 and sR-989262 shown to decrease LPS-induced IL-8 secretion in human gastric adenocarcinoma (AGS) cells [50]. Moreover, a study involving sRNAs from periodontal pathogens revealed a consistent pattern among the bacteria: synthetic sRNA oligonucleotides from each pathogen, when introduced into Jurkat T cells, led to a reduction in anti-inflammatory cytokines such as IL-5, IL-13, and IL-15 [32]. Additionally, Gu *et al.* demonstrated that *Salmonella enteritidis* , upon entry into intestinal epithelial HT-29 cells, released RNAs that were processed into mature forms by host cell mechanisms such as Sal-1, uncovering a novel mechanism for *Salmonella* to evade host immune defense by reducing Sal-1 levels [51].

4.3. Bacterial sRNAs as virulence factors in infectious diseases

Throughout the prolonged interaction between pathogens and their hosts, pathogens can modulate their gene expression by sensing host signals, such as temperature, pH, oxygen concentration, and osmolarity, to adapt to the internal environment of the host and survive within it [52]. sRNA-mediated regulation of gene expression enables pathogens to respond more rapidly to changes in the external environment. Upon pathogens entry into the host, the detection of changes in the host microenvironment allows for the modulation of virulence gene expression via sRNAs, enhancing the pathogens’ survival capabilities within the host and facilitating its invasion and pathogenicity [53]. To date, a variety of sRNAs associated with bacterial virulence and pathogenicity have been identified. These sRNAs serve as critical components of the bacterial virulence regulatory network, finely tuning bacterial virulence and pathogenic mechanisms at the post-transcriptional level [54]. In Enterohemorrhagic *Escherichia coli* (EHEC), the sRNAs GlmY and GlmZ are known to form a regulatory cascade that significantly influences the expression of key virulence factors. These sRNAs act in concert to modulate the stability and translation of mRNAs encoding these factors, thus directly impacting the pathogen's ability to cause disease [55]. In *Helicobacter pylori*, a pathogen linked to the development of gastric cancer, the sRNA HPnc4160 has been identified as a critical player in the pathogen’s adaptation to the gastric environment. This sRNA exhibits differential expression in bacterial isolates derived from patients with gastric cancer compared to those from patients without cancer, suggesting a potential role in modulating disease severity and progression. Such differential expression highlights the role of sRNAs in pathogen adaptation and virulence, underscoring their potential as targets for therapeutic interventions [56].

4.4. Prospects of bacterial sRNA applications in diseases: bacteremia

Bacteremia is a systemic infectious disease characterized by the entry of pathogenic bacterial into the bloodstream, where they grow, multiply, and produce toxins. Bacteremia is associated with high mortality rates [57–59], which are strongly influenced by key risk factors such as the patient's underlying disease [60,61], polymicrobial sepsis with mixed infection [62], antimicrobial resistance [63]. Culture-based methods remain the gold standard to identify the causative microorganism in sepsis. It is recommended to collect a minimum of two sets of blood cultures, comprising both aerobic and anaerobic cultures, with each bottle containing 10–20 mL of blood. This process should be conducted following thorough skin disinfection procedures [64]. However, blood cultures are not 100% sensitive. This may be due to recent exposure to antibiotics or difficult-to-culture microorganisms such as Borrelia, Bartonella, Coxiella or Rickettsia [65]. Furthermore, blood cultures are associated with the drawback of prolonged detection cycles, leading to delayed and precise clinical diagnoses. Molecular assays are increasingly deployed in bacteriology laboratories as rapid alternatives to culture-based methods [66]. Here, we propose sRNAs could serve as novel tool for the early detection of bacteremia. Since specific sRNAs are unique to certain bacterial strains, their detection in a patient's blood could not only confirm the presence of bacteremia but also help identify the specific bacterial pathogen involved, thereby enabling targeted treatment. Moreover, the levels of certain bacterial sRNAs in the bloodstream could potentially be used to monitor the efficacy of antibiotic treatment in real-time, providing a dynamic way to assess patient response to therapy. However, the application of bacterial sRNAs in the clinical management of bacteremia is still in early stages, and much research is needed to translate these concepts into practical medical solutions. One of the main challenges is distinguishing between sRNA signatures that indicate bacteremia and those found in patients with localized bacterial infections without bacteremia. This differentiation of two situations is crucial for appropriate treatment decisions, as bacteremia can lead to systemic infections and requires immediate intervention. Developing diagnostic tests that are both highly sensitive (able to detect low levels of bacterial sRNAs indicative of early-stage bacteremia) and highly specific (able to accurately identify the bacterial species causing the infection and distinguish between bacteremia and other conditions) is also a significant challenge. The risk of false positives or negatives can significantly affect patient management and outcomes. Moreover, there is a need to establish standardized baseline levels of bacterial sRNAs that are indicative of bacteremia. This involves determining what levels of specific sRNAs are considered normal in a healthy individual, as well as the threshold levels that suggest a bacterial infection has progressed to bacteremia. Variability in sRNA expression among different bacterial species and strains, as well as differences in patient immune responses, complicates this task.

5. Conclusion

Bacterial sRNAs are ubiquitously present in the microbial world, playing pivotal roles in bacterial physiological functions, communication between hosts and microbes. The discovery of these molecules in bacterial EVs, particularly their detection within the host, reveals their potential significance in host-microbe interactions. These vesicles, which carry a variety of molecular components, are now recognized as key facilitators of the interactions and communications between bacteria and human host. The detection of bacterial sRNAs in human body fluids such as plasma highlights their potential role as novel biomarkers for various diseases and infections. Moreover, the role of sRNAs in modulating host immune responses offers a new perspective on the complex dynamics of host-pathogen interactions. However, significant challenges remain in harnessing the full potential of bacterial sRNAs. Key among these are the development of sensitive, specific methods for detecting sRNAs in human fluids, and a deeper understanding of the intricate interactions between these RNAs, their bacterial sources, and the human host. As research progresses, it is anticipated that bacterial sRNAs will emerge as crucial tools in the diagnosis and treatment of infectious diseases, enhancing our capacity to combat microbial threats and safeguard human health.

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Conflicts of interests

The authors declare no conflicts of interest.

Authors' contribution

Conceptualization, Qipeng Zhang and Linyao Fan; methodology, Wenjiong Xie and Chang Xu; software, Yang Lu; validation, Qingyi Shen, Jiao Cang, and Yaoyao Ma; formal analysis, Wenjiong Xie; investigation, Linyao Fan; resources, Jiao Cang, and Yaoyao Ma; data curation, Qingyi Shen; writing—original draft preparation, Linyao Fan; writing—review and editing, Linyao Fan; visualization, Chang Xu; supervision, Qipeng Zhang; project administration, Qipeng Zhang; funding acquisition, Qipeng Zhang. All authors have read and agreed to the published version of the manuscript.

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