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Review

Extracellular RNAs and Extracellular Vesicles: Inception, Current Explorations, and Future Applications

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ABSTRACT - In addition to the ubiquitous roles of cellular RNA in genetic regulations, gene expression and phenotypic variations in response to environmental cues and chemotactic signals, the regulatory roles of a new type of RNA called extracellular RNAs (exRNAs) are an up-and-coming area of research interest. exRNA is transported outside the cell through membrane blebs known as membrane vesicles or extracellular vesicles (EVs). EV formation is predominant and conserved among all microbial forms, including prokaryotes, eukaryotes, and archaea. This review will focus on the three major topics concerning bacterially derived exRNAs, i.e., 1) the discovery of exRNA and influence of extraneous RNA over bacterial gene regulations, 2) the known secretion mechanism for the release of exRNA, and 3) the possible applications that can be devised with these exRNA secreted by different gram-negative and gram-positive bacteria. Further, this review will also provide an opinion on exRNA- and EV-derived applications such as the species-specific exRNA markers for diagnostics and the possible roles of exRNA in probiotics and the epigenetic regulations of the gut microbiome.

Keywords: exRNA, Extracellular vesicles, Secretory mechanisms, Molecular pathogenesis, Diagnostics

Apart from the vital roles of mRNA, tRNA, and rRNA in central dogma and protein synthesis, the intricate regulatory roles played by other coding and non-coding RNAs like small RNA (sRNA), micro RNA (miRNA), and silencing RNA (siRNA)[1-6]. exRNAs and secretory RNAs (sRNAs) have attracted significant attention[7-9]. Several new types of RNAs and their intuitive roles have been identified in the last decade, showing their relevance with the cell architecture and the host-pathogen interactions, including molecular pathogenesis and persistence of infections. However, the discovery of extracellular nucleic acids (exNA) and different secretory systems, especially by the pathogenic microbes, has provided new insight about unknown molecular events that may entail various biological events associated with these exNA like cell homeostasis, molecular pathogenesis, genetic transfer of information between cells, molecular communications, etc..

The exNA, comprising RNA transcripts or gene fragments, is ubiquitous in the environment released from dying and decaying biological matter, including active secretions of living cells. In this regard[10], reported the role of extracellular DNA (exDNA) in biofilm formation and its relevance in mediating antibiotic resistance. Nishimura et al. (2003) demonstrated the active release of exNA containing double-stranded DNAs and single-stranded RNA molecules

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(similar to tRNA) in marine bacterium *Rhodovulum sulfidophilum*. Bacteria present in biofilms or soil sediments use exNA as their energy source under stress conditions[11], potentially involving horizontal gene transfers[12]. Apart from such classical examples on exNA, there was also a report on the existence of heat-stable RNA fractions in complex biological media (meRNA) used for bacterial and animal cell cultivation and the involvement of meRNA in altering the proteome of the bacteria[13]. The circulating exNA, found in milk, plasma, amniotic fluids, bronchial secretions, body fluids, etc., are proposed as etiologies for diseases like cancer in the field of biomedicine[14]. Therefore, identifying characteristic or specific exRNA or exDNA provides information on cell specificity and tumor location in the tissue[15]. *In this order, s*ecretion of RNA in prokaryotic organisms has recently gained researchers' exclusive attention working in molecular pathogenesis, disease diagnostics and biomarker development, probiotics, and researchers working on CRISPR technology[8,9]. Bacterial RNA and DNA-RNA hybrids elicit innate immune responses in the host organism[16,17], indicating physiological cell-viability. Secretory prokaryotic mRNA molecules are potential targets for pathogen-associated molecular patterns of the host immune system, implicating secretory RNA's potential in aiding pathogenesis[18]. At this outset, this review is inspired by several recently published research and review articles on exRNA and their proposed multifold association in the microbial milieu. This review will focus on exRNA and its origin, secretion mechanisms, and types of different reported exRNA. This review will also focus on exRNA as a target for further research in biomedical applications and probiotics.

Origin of microbial exRNA

The secretion of microbial exRNA by bacteria was observed in studies dating back to as early as 1989. One of the earliest reports showed RNA in membrane vesicles (also referred to as blebs) of *Neisseria gonorrhoeae*[9,19]. However, the secretion of microbial exRNA's by bacteria was not explicitly noted and mentioned till 2003, when a study in the photosynthetic bacteria *Rhodovulum sulfidophilum* reported the secretion of exRNAs. Further analysis of the RNA using two-dimensional gel electrophoresis showed that these secreted RNAs had sizes comparable to t-RNA molecules[20]. A few years down the line, a study by the same group characterized these extracellular RNAs from the same bacterial species. *Rhodovulum sulfidophilum* being a marine autotrophic microbial organism, experiments in both light and dark conditions showed that the amount of extracellular RNA was produced by the bacteria had a trend that was similar to the curve of bacterial growth. The production of extracellular RNAs was initially low, followed by a spike in production before a significant production decrease took place. Analysis of band intensity obtained from denaturing gel electrophoresis was used to characterize the production of the extracellular RNAs because quantification using spectrophotometric methods proved unreliable. This was probably due to the presence of proteins and polysaccharides, which were co-secreted with nucleic acids. The major bands of RNA obtained after electrophoresis was between the range of 70 bp and 300 bp. Further cloning and sequencing of the RNAs revealed that the secreted RNAs consisted of an assortment of fragments of 16s rRNA and 23s rRNA and a potpourri of tRNAs. Analysis of the secreted exRNA sequences' composition revealed that it was similar to intracellular RNA molecules present inside the bacteria[21]. In a study using *Pseudomonas aeruginosa*, it was suggested that these bacterial nucleic acids could be transported to other bacteria via membrane bound vesicles, which helps to transport this either through a periplasmic or an exogenous route. These membranes bound extracellular vesicles were hypothesized to protect the nucleic acid from degradation[22]. In a clinically relevant aspect, live cultures of *Mycobacterium tuberculosis* were observed to induce apoptosis in the monocytes through the secreted exRNA. With convincing circumstantial evidence, it was hypothesized that these bacteria secrete certain specific molecules that result in apoptosis in monocytes. On analysis of the samples, it was found that these bacteria secrete certain exRNA molecules, which are composed of both tRNA's and rRNA's that induce apoptosis. This was one of the earliest papers that showed the relevance of exRNA in host-pathogen interactions and to modulate the killing of host immune cells in the human body[23]. Over the past few decades, we have learned a lot more about these ex RNAs and their diversity with the rapid advances in sequencing technologies and the advent of next-generation sequencing techniques, with membrane vesicles being the primary means of nucleic acid secretion by bacteria. Sequencing of RNA from extracellular vesicles of the marine bacterium *Prochlorococcus spp* revealed the presence of a wide diversity of RNA molecules in the vesicles[7,9]. RNA-containing extracellular vesicles have been located in a wide range of gram-positive bacteria and gram-negative bacteria[8]. These exRNAs have been characterized in several species, including *Pseudomonas aeruginosa*, *uropathogenic Escherichia coli* str. 536, *Vibrio cholera* str.01, group A, *Streptococcus sp*., *Aggregatibacter actinomycetemcomitans*, and *Porphyromonas gingivalis*[25-27]. In recent years there have been standardized protocols for isolating, studying, and characterizing these exRNA's from microbes. The protocol consists of three main steps i) Isolation of Membrane vesicles ii) Extraction and purification of RNA iii) Characterization of the RNA using RNA sequencing. The process of isolating pure

membrane vesicles and preventing RNA samples from getting contaminated is highly finicky[28,29]. Characterization of the extracellular vesicles has also been identified as an important part of studying the transmission of these RNA molecules and fragments across species and different communities. Exosomes isolated from different sources, including mammalian sources, have been observed to be uptaken by bacteria. A variety of techniques, including transmission and scanning electron microscopy and western blotting, have been used to study these membrane-bound vesicles[30]. With a plethora of such techniques and tools available to study the exRNA and its secretions, an ongoing research question is how these RNA molecules are secreted? Are these secretions selective to specific RNA molecules?. The following section explains the widely accepted theories and current experimental evidence behind the active secretion of exRNA by different bacterial species.

exRNA excretion/secretion mechanism and intercellular communications

There has been a surge with evidence accumulating towards the vesicles mediated secretions and reports on DNA vesicles since 2005[31]. This specific study provides interesting perspectives on the relevance of RNA vesicles in bacterial survival and adaptation. Nevertheless, it is not new that vesicle-mediated delivery of toxins, virulence proteins, and nucleic acids is highly efficient in Gram-negative pathogens like *Enterotoxigenic E. coli, Salmonella,* and *Pseudomonas species*. Vesicles are also reported to be protective of virulence factors from host destruction and help in immune evasion[31,32], thus providing a distinct advantage to the pathogen to establish its virulence in a host. These blebs are known to be actively produced during the mid-log phase to the end of the log phase at an O.D approximating 0.5 to 0.8[33-38]. Furthermore, as would be expected for the cargo, the kinetic profile of exRNA also matches mainly the abundance of tRNA, rRNA, and copies of mRNA abundant during the mid-log phases of bacterial growth, the active bacterial growth timepoints. Further, in 2015, Kim et al.'s[8] study showed the membrane vesicle transport machinery in the transport of proteins, nucleic acids, and metabolites between bacterial cells. It appears to be important in survival and colonization[8]. Further, the characterization by EM and other high-resolution microscopy techniques elucidated the size of membrane blebs and membrane vesicles to be 30 nm to 150 nm in sizes. Also, as

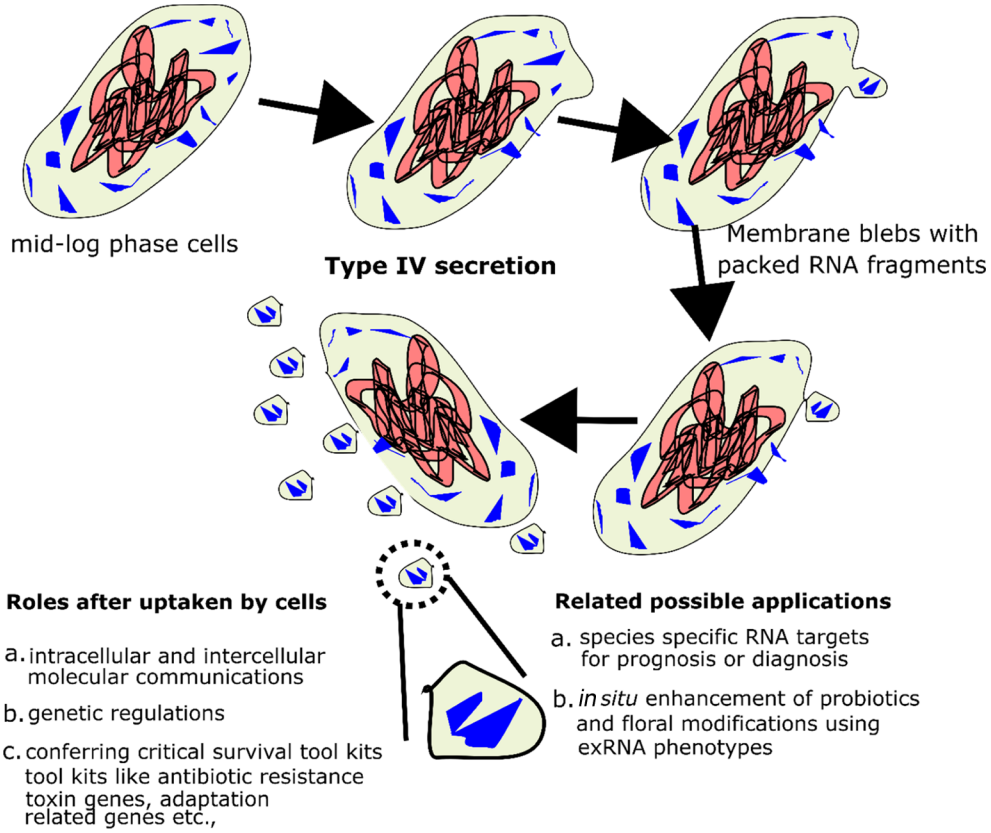

Fig. 1. The schematic figure of exRNA exported in membrane vesicles using type IV secretion systems. Note: DNA/nucleolus - pink, cytoplasm - green, RNA - blue. The small circular units with blue patches represent the EV. While it could be different biomolecules, here we are showing only exRNA.

additional proofs, these membrane vesicles were not a typical byproduct of cell lysis but secreted by active secretion mechanisms like type IV secretion mechanisms[25]. These membranes bound vesicles that arise from the budding that occurs internally in the plasma membranes are then secreted by the bacteria. Also, there are circumstantial evidence that type IV membrane vesicles secretion is discrete, closed outer membrane blebs produced by actively growing cells and are not as a result of cell lysis. Such secretions are also reported in most of the pathogenic and non-pathogenic gram-negative strains of microbes for survival[26] (Fig. 1). In the case of virulence structures, these are used explicitly for transporting virulence factors into the host as given by various biochemical and functional assays for studying vesicular transport. This phenomenon is observed unanimously in many gram-negative bacteria when grown in any environment like liquid culture, solid media, and even during biofilm formation, with 0.2-0.5% of OM protein of the bacteria extensively used for the secretion process[39]. Further, the vesicle's maximum production is during the late log phase and mid-log phase, as documented in *E. coli*, *Vibrio cholera, B. melitensis*. MVs are abundant in the sites of cell division[39,40], with a pathogenic form of bacteria secreting excess of these vesicles compared to the non-pathogenic counterpart, i.e., approx. ten-fold increase in secretion compared to the non-pathogenic forms of *E. coli* [40]. These results support that the bacterial pathogens have usurped the phenomenon to disseminate virulence factors, and the phenomenon is common to both intracellular and extracellular pathogens. Also, reports are suggesting the transport of beneficial materials like genetic material, nucleic acid, antibiotics resistance genes, DNA protein complexes, single-stranded DNA complexes, nucleases resistant DNA into the host, including prokaryotes and eukaryotes, as a means to conform essential genetic information and thus transform the bacteria in a stressed environment[43-45] in *P.aureginosa, Agrobacterium tumefacient,* etc. The vesicles could impact the establishment and longevity of a bacterial infection[31]. While much remain to be explored, and such extracellular vesicles and their secretion mechanisms, more insights on their emergence could be studied by recent advancements in micro and nanofabrication techniques using microfluidic devices with constrained microfluidic geometries[33]. The study of such phenomenon's at single cell levels could give better insights to decipher further the molecular build-up, leading to better use of such novel secretion phenomenon to employ in effective applications and to answer the unknowns like exRNA-EV secretion molecular events.

Fate of exRNA packed in EVs

As a universal messenger with critical functions, ability to be associated with wide variety of proteins in the form of RNA-binding proteins[34], ability to be associated with membranous molecules of the cells[35], ability to be transferred into the recipient with functional roles[35] plots a multifold role of exRNA secreted via EV. There are several potential roles that the exRNA continuing EVs could impart to the host cells, grouped in to three categories namely -

a. conferring genetic traits;
b. molecular communications; and
c. gene expression regulation.

Excreted or secreted EVs could also be up taken by different classes or domains of organisms, like eukaryotes, prokaryotes, and other cross-species among eukaryotic vesicles[45]. Albeit not very relevant to the bacterial cells, exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells[45]. Such membrane vesicles have been referred to in the possible transfer of adaptation and survival-related tool kits in harsh environmental conditions to the up taking hosts in the form of secreted vesicles containing specific types of proteins, immune cell evasions factors, immune cell suppression factors, response to stress factors and transfer of virulence traits. A detailed review by MacDonald and Kuehn, 2012 explains the offensive and defensive roles played by membrane vesicles in bacterial survival across both gram-positive and gram-negative species. A wide repertoire of RNA's including small RNA's (30 nts - 250 nts), CRISPR RNA's, tRNA's, mRNA's and rRNA's have been reported in various microbes[9]. The various types of exRNA's are packed into extracellular membrane-bound vesicles that protect the RNA from external influences and degradation from endonucleases and exonucleases activity like RNase's. The localization of these RNAs in the membrane-bound extracellular vesicles takes place through selective mechanisms that involve specific motifs present in the RNA's and with the help of certain RNA binding proteins. However, the mechanisms behind RNA sorting into extracellular vesicles remain relatively uncharacterized. However, a question that is not well understood is if the secretion process is selective to certain types of genes or any random packing of cytoplasmic contents with RNA transcripts. Further studies must focus on understanding the specific molecular mechanisms behind the sorting of RNA molecular cargo in these vesicles. The secreted vesicles could then be transferred to other microbes or eukaryotic cells. These membranes bound vesicles either fuse with the outer membrane of target cells or are internalized by endocytosis. On fusing with the membrane, the RNA molecules are released into the cells. In contrast, once the membrane bound vesicles are internalized, the RNA gets released inside the cells via endosomal escape. The RNA

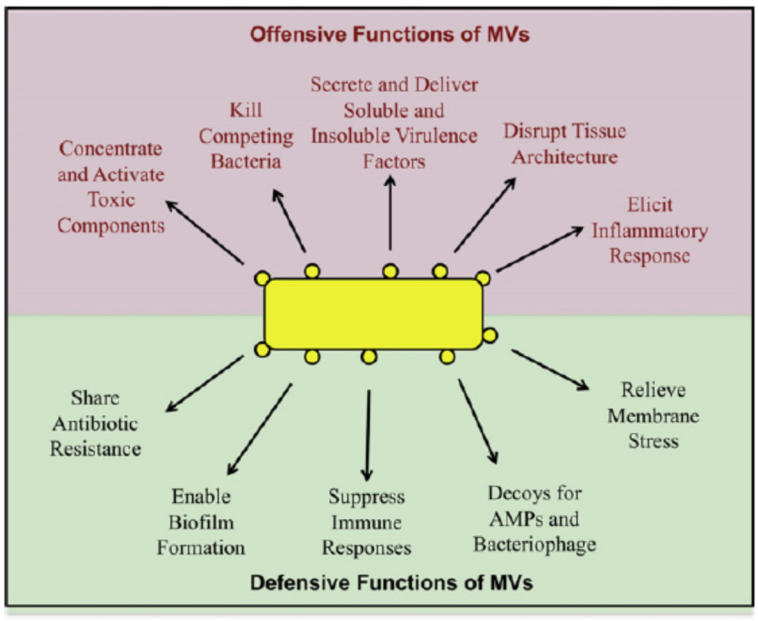

Fig. 2. Roles of membrane vesicles in offensive and defensive functions that aid in the colonization and survivability of the bacteria. Figure reproduced from MacDonald, and Kuehn 2012[55]. Note: the above functions are not restricted to EVs that contain RNA only, but overall, to different types of biomolecules that can be packed in the MVs.

molecules can then bind to their specific targets and perform their functions like modulating the gene expression[9,46,47]. Various micro RNA, like small RNA molecules, have been identified and characterized by periodontal pathogens. The pathogenic bacteria *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, and *Treponema denticola* were found to secrete tiny RNAs via a membrane-bound vesicle. These were predicted to have targets inside human cells and were hypothesized to regulate immune system-related genes' functioning and expression[47]. A recent study with micro RNA sized exRNA's from the periodontal pathogen *Aggregatibacter actinomycetemcomitans* delivered in membrane-bound extracellular vesicles was found to regulate the expression of TNF-alpha in macrophages derived from humans. Further studies using mouse models showed that these OMVs helped permeate the blood-brain barrier[48]. Similarly, there have been multiple reports of cellular communication with eukaryotic cells using membrane vesicle bound RNA's observed in other bacteria (especially pathogenic bacteria) like *Pseudomonas aeruginosa*[49] and *Leishmania sp*.[50]. Most of the studies that elucidate intracellular communication have shown how this membrane-bound vesicle containing extracellular RNA gets internalized in eukaryotic host cells and how they regulate functions and communicate with these cells. Further studies are required to understand whether or not these small RNA molecules have an impact on microbial ecology. Further studies should also focus on the uptake of outer membrane vesicles containing microbial RNA is on interspecies and species communication. Vesicle encapsulated exRNA's from mammalian sources like milk were found to modulate the expression of genes and promote the growth of two probiotic species *Escherichia coli* K-12 MG1655 and *Lactobacillus plantarum* WCFS1[51]. Various small RNAs have also been found in quorum sensing regulation in two *Vibrio spp*[52]. Small RNA's which do not code for proteins have played an important part in the regulation of gene expression in many microbes and have played an important part in major physiological processes in bacteria like regulation of RNA binding proteins and regulate the activity of several key enzymes[53]. Regulatory RNAs that do not code for proteins have been identified in bounty in extremophilic communities using meta-omics approaches[54]. These suggest that these RNAs can play a role in community signaling. Hence, studying the role of these encapsulated exRNA's in intra and inter-species gene and protein regulation and modulation can help open up new frontiers in microbial ecology and help design new therapeutic interventions.

Also, recently, MVs mediated transfer of proteins and nucleic acids, particularly RNA, has been shown to be prominent in biofilm-producing foodborne pathogens like *Salmonella spp, Campylobacter spp, Pseudomonas spp, E. coli, and H. pyroli* (Table 1, Begić and Josić (2020)[56]). While numerous reports target the transport of toxins and other small molecules through MV, very few or no compelling evidence that have been shown that RNA

Table 1. List of exRNA-EVs and their description and functional roles

Bacteria	Description of exRNA in EVs	exRNA-EV functions	Reference
Aggregatibacter actinomycetesmcomitans	sRNAs detected by RT-qPCR and Northern Blot	n.d.,	
Escherichia coli	15-40 nt, primarily derived from tRNA and rRNA cleavage products	n.d.,	
Mycobacteria smegmatis		n.d.,	
Neisseria gonorrheae		n.d.,	
Porphyromonas gingivalis	cDNA derived from protein coding and 16S RNA detected by RT-qPCR	Indicator of bacterial metabolic state at time of lysis; sRNA transferred to host epithelial cells and reduced LPS-stimulated MAPK signalling pathway	9
Prochlorococcus marinus	Mapped to 95% of open reading frames	n.d.,	
Pseudomonas aeruginosa	15-45 nt; many with predicted stable secondary structure; primarily derived from coding regions; enriched for SOS-stress response and pyocin genes	n.d.,	
Streptococcus pyogenes	rRNA and RNA primarily derived from coding regions	n.d.,	
Streptococcus sanguinis	sRNAs detected by RT-qPCR	n.d.,	
Vibrio cholerae	Primarily derived from intergenic regions	n.d.,	
Treponema denticola	sRNAs detected by RT-qPCR and Northern blot	n.d.,	
Campylobacter spp	n.d.,	n.d.,	56
Pseudomonas spp	n.d.,	n.d.,	56
E. coli	n.d.,	n.d.,	56
Helicobacter pyroli	Various type of cargo	Possible biofilm formation	56
Pseudomonas aeruginosa	Membrane vesicle bound RNA fragments	n.d., possible intracellular comminucation	57
Leishmania sp.	Membrane vesicle bound RNA fragments	n.d., possible intracellular comminucation	50

n.d., not determined.

molecules are secreted vial MVs among food borne pathogens in relevance to pathogenesis, or such observations are yet to come, according to our broad literature review. Below is a cumulative list of different functions and descriptions of bacterial derived exRNA-EVs.

Perspective roles of exRNA in biomedical and food technology applications

The research in exRNA and circulating RNA species is a promising effort in the prognosis and diagnosis of cancer and is a demonstrated diagnostic phenomenon particularly in terms of extracellular nucleic acids. Also, Detection of circulating RNA in cancer patients is advantageous over DNA based detection as the RNA based detection can indicate the tumor and tissue specificity of it as the RNA is specific to the cell and its location[58]. With the current knowledge on EVs and the association of RNA molecules in EVs, this could be a promising avenue for diagnosing exRNA from cells towards pathogen detection particularly for identification of novel cancer biomarkers. However, these are easier said than done because there are many loopholes to understand why and how such exRNA are secreted out, their selectivity or the lack of it during packing, and consistent packing of specific RNA targets could unveil several diagnostic tools that could be non-invasive. Further, conservation of exRNA fragments in packaged food and their stability could shed light on using exRNA fragments in foodborne pathogen diagnosis. Since RNA secretion is a ubiquitous phenomenon, it is reported across a wide range of gram-negative and gram-positive species. Such diagnostic themes have very high value return on investment when coupled with flexible biosensors for point of care tools[59,60] and other microfluidic detection techniques[61].

Since several beneficial microbes' harbor host enteric regions, there is a very significant possibility that such exRNA secreted via EVs could be used for intracellular communications among the microbial flora in the intestines. This could have added-values in the preparation of probiotics with synthesized exRNA fragments, which would bring beneficial effects to the flora already existing in a

person. While the ideas mentioned above and principles are more theoretical, and there are seldom clear proof and lack of convincing evidence, such research would shed light on the role of exRNA in enhancing microbial flora and probiotics effects in humans.

Conclusions

The inception of exRNA was a relatively old discovery. However, it has been extensively researched in the recent decade due to its ubiquitous nature and the multi-fold relation that exRNA has in modulating molecular events at cellular and sub-cellular levels. While there is accumulating evidence on how membrane blebs could export exRNA or, in general, exNA and if this process is species-specific, the use of exRNA from bacteria for diagnostics is much intriguing. As a result, the roles are intriguing to explore the phenomenon of exNA release in clinical isolates to understand the unknown molecular events of host-pathogen interfacial interactions that can be used as plausible diagnostic targets.

국문요약

유전적 조절, 유전자 발현 그리고 환경적 단서, 화학적 신호에 대응하는 표현형 변이에서 세포 RNA는 ubiquitous 역할 이외에도 세포 외 RNA(exRNA)라 하는 새로운 형태의 RNA는 추후 연구의 방향을 제시한다. exRNA는 membrane vesicles 또는 세포 외 소포체(EV)로 알려진 membrane blebs를 통해 세포 외부로 운반된다. EV의 형성은 원핵생물, 진핵생물, 고세균을 포함한 모든 미생물군에 우세하게 보존되어있다. 본 리뷰는 세균 유래 exRNA에 관해 세가지 주제에 초점을 두었다. exRNA의 발견과 박테리아 유전자 배열에 대한 외부 RNA의 영향, b. exRNA의 분비기작을 통한 방출, c. 다른 그람음성 및 그람양성균에 의해 분비되는 exRNA로 고안될 수 있는 응용 가능 분야이다. 본 리뷰에서 장내 미생물군의 probiotics 및 후성유전학적 규제에서 본 exRNA와 exRNA마커와 같은 EV 파생 응용프로그램에 대한 의견을 제공할 것이다.

Conflict of interests

The authors declare no potential conflict of interest.

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