Integron/gene cassette metagenome in marine environments, exploring and applications.

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A review article

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Abstract

Recently, integron gene cassette metagenome has been discovered as a class of mobile DNA elements that play a role in the functional genome of marine bacteria. This importance of integron was extended from the original idea that integron is the main key for evolution and adaptation of pathogenic bacteria against antibiotic attacks. In this review, we highlighted on the diversity, function and applications of integron/gene cassette metagenome in marine environments. Each environment has specific selective characteristics that could shape the integron gene cassette metagenome, to be an environment adaptive DNA tool. This idea led us to innovate synthetic integrons with wide biotechnological applications.

١. Introduction

The genomics era has clearly indicated that a large proportion of marine bacterial genes have been acquired by horizontal gene transfer [Ochman et al., ٢٠٠٠]. This type of gene transfer is facilitated by a number of genetic elements, including plasmids, transposons, and integrons. Traditionally, most attention has focused on plasmids and transposons [Smalla et al., ٢٠٠٠]. However, since integrons have recently been demonstrated to occur in the genome of diverse marine uncultured bacterial species and integron integrases are recoverable from various marine environmental samples [Elsaied et al., ٢٠٠٧; Rodríguez-Minguela et al., ٢٠٠٩]; we reasoned that integrons are widespread in marine environments.

Integron is DNA element described in pathogenic and environmental bacteria, first recognized and named by Ruth Hall and Hatch Stokes in ١٩٨٩ [Stokes and Hall, ١٩٨٩]. Integron carries varieties of functional expressed genes, in the form of cassettes, which play an important process in shaping bacterial genomes over evolutionary time scales. In particular, in the context of the short-term evolution of bacterial genomes, mechanisms of non-homologous recombination

are known to be crucial to adaptation in extreme environments. The most obvious example of this is the extraordinary ability of pathogens to acquire multiple antibiotic-resistance genes in historical times, a feature characterizing only integron metagenome. Recently, integron was found to have the flexibility to acquire and expel genetic elements adaptive to several stresses, other than antibiotics, in marine environment [Elsaied et al., ٢٠٠٧; Koenig et al., ٢٠٠٩]. This integron unique feature created a system that provides for an enormous pool of known and unknown adaptive genes to be mobilized, rearranged, and disseminated amongst marine bacteria. Indeed, the reservoir of adaptive genes capable of being mobilized as yet has no known upper limit but must, at the very least, number in the thousands and is probably orders of high magnitude. As a result, integron system can greatly influence marine bacterial diversity and adaptation in ways that other DNA tools cannot.

In order to understand the integron metagenome contribution to the evolution and adaptation of marine bacteria, we looked at extreme marine environments where the bacterium exposes to various selective environmental pressures, which stimulate integron diversity and abundance. We described the diversity and shape of integron metagenome in two types of extreme marine environments. The first is deep-sea hydrothermal vents, environments completely away from anthropogenic activities, where both free-living and symbiotic bacteria expose to different selective natural pressures such as high temperature and toxic gases erupted from the vents [Cherry et al., ١٩٩٢]. The second constitutes the urban marine sediments, which are exposing to a high load of different human industrial waste pollutions.

٢. Integron structure in marine environment

Integron is a site-specific recombination system. The ideal integron consists of three DNA structures, which are integrase gene, *intI*, attachment site, *attI*, and integrated gene

cassettes (**Fig.** ١). The integrase gene, *intI*, encodes the enzyme integrase, belonging to the tyrosine recombinase family, which is responsible for integration of gene cassettes inside integron [Esposito and Scocca, ١٩٩٧].

Like other enzymes, integrase, IntI, has specific motive catalytic sequence, which encodes the conserved amino acid residues [RHRY] found in two sequence boxes [I and II] located in the carboxylic half of the integrase protein [Nancy and Roy ٢٠٠١]. *intI* was identified in marine integron metagenomes through PCR amplification and sequencing of only fragments from the *intI* terminal part, which encodes the carboxylic half of the expressed enzyme. Molecular culture-independent studies obtained *intI* sequences of size ٧١٤ bp (٧٠ % of full ideal *intI* sequence ~١٠٢٠ bp) from varieties of free-living and symbiotic hydrothermal vent bacterial metagenomes [Elsaied et al., ٢٠٠٧, ٢٠١١; ٢٠١٣], while only ٤٩٢ bp of *intI* were sequenced from urban shore and Antarctic marine sediment metagenomes [Minguela-Rodrigues et al., ٢٠٠٩]. *intI* sequences recovered from marine metagenome contained some of the catalytic conserved *intI* regions founded in clinical and cultured isolates [Grainge and Jayaram, ١٩٩٩], implicating the global consensus motive structure of *intI*.

The promoter, called *Pc*, located upstream to *intI*, [Collis and Hall, ١٩٩٥] and responsible for expression of integrated gene cassettes, has yet to be characterized experimentally in marine integron metagenome.

The attachment site, attI, is the integron-associated recombination site, where the gene cassettes are captured by integrons [Collis et al., ٢٠٠٢] (Fig. ١). Thirty three diverse attI-like sequences have been detected in marine integrons, and most contained an integrase-binding simple site, but lacked a pair of direct repeat sequences as found in attI of known integrons in pathogenic bacteria (Partridge et al., ٢٠٠٠; Elsaied et al., ٢٠١١, ٢٠١٣).

The units of insertion into integrons are mobile gene cassettes. A gene cassette is an independently mobilizable element that generally contains a promoterless ORF and an intI-recognizable recombination site called the *attC* [Stokes et al., ٢٠٠١]. In several integrated gene cassettes recorded from marine metagenomes, *attC* has been considered as part of the ORF, while in others it is located downstream to the ORF [Elsaied et al., ٢٠٠٧; Koenig et al., ٢٠٠٨, ٢٠٠٩]. The *attC* sites have diverse sequences and lengths in both clinical and environmental isolates but share common features including about ٢٥ bp at each end that conform to consensus sequences [Nield et al., ٢٠٠١; Stokes et al., ١٩٩٧, ٢٠٠١]. The *attC* consensus regions are imperfect inverted repeats of one another, and each comprises a pair of inversely oriented integrase-binding domains, separated by a spacer of ٧ or ٨ bp [Hall et al., ١٩٩١] (**Fig. ٢**). The recombination crossover has been localized between the G and first T of a seven-base core site (region ١R in **Fig. ٢**) located in the right-hand simple site. Recombination activity of *attC* from deep-sea integron metagenome has been measured experimentally (Elsaied et al., ٢٠٠٧). The lengths of *attC* recorded from marine metagenomes were ranged from ٥٩ bp to ٢٣٠ bp, an implication for enormous diversity of *attC* in marine environment [Elsaied et al., ٢٠١١, ٢٠١٣; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨].

There is no obvious evidence for the origin of marine integron metagenome. However, integron is an old genetic element in the evolution of bacterial genome and not related, in origin, to the short history of antibiotics. This concept is confirmed by the discovery of integron in deep-sea bacteria, representing the oldest form of bacterial genome in our biosphere and completely isolated from any effect of antibiotics [Elsaied et al., ٢٠٠٧]. On the other hand, lateral gene transfer through bacterial conjugation may help in the evolutionary spreading of integrons

between clinical pathogenic and marine bacteria through disposable wastes of human into the marine environment [Wright et al., ٢٠٠٨; Koenig et al., ٢٠٠٩].

٣. Diversity of integrase gene, *intI*, as a marker for integron diversity in marine environments

Two features supported studying the diversity of integron based on integrase gene. The first feature is the common existence of integrase gene as the backbone of any integron recovered from both of clinical and environmental bacteria. The second feature is conservation of the integrase motive sequence in almost all recovered bacterial integrons [Nancy and Roy, ٢٠٠١].

Originally, three major groups (class ١ to ٣) of integron integrase are highly prevalent in the clinical scene [Mazel et al., ١٩٩٨]. In most of the cases, these three classes of integrase have also been reported to catch up almost exclusively gene cassettes encoding antibiotic resistance functions [Mazel, ٢٠٠٦]. All together, these traits have led to their designation as mobile clinical integron integrases [Gillings et al., ٢٠٠٨]. Although integrons have been traditionally classified according to the percent identity of the nucleotide or predicted amino acid sequence of their respective integrase genes, several structural features and differences in abundance patterns have been identified and distinguished classes ١ to ٣ [Biskri et al., ٢٠٠٥; Xu et al., ٢٠٠٧].

Class ١ integron integrase, *intI١*, is the most widely studied variant and is typically linked to replicative *Tn٢١* transposons, which appear to contribute to their extensive distribution [Nandi et al., ٢٠٠٤]. The diversity of class ١ integron has been extended beyond clinical field. The relative *intI١* abundance, based on qPCR, was high in bacterial metagenomes isolated from polluted estuary and shore sediments [Gillings et al., ٢٠٠٨; Wright et al., ٢٠٠٨], implicating the role of lateral gene transfer in spreading of this class in both clinical and marine bacteria.

Recently, we discovered class ١ integrase gene in a deep-sea symbiont living within a gutless clam, *Calyptogena* sp. (accession no. AB٢٧٣٧٢٧) [Elsaied et al., unpublished data], Sagami bay, Japan, and recording amino acid identity ٨٧٪ with that of *Escherichia coli* (**Fig. ٣**), a feature supporting the concept that this class has an ancient phylogenetic lineage in bacterial genome.

In contrast to class *intI١*, class ٢ integrase, *intI٢*, is routinely associated with non-replicative *Tn٧* transposons [Ahmed et al., ٢٠٠٦; Barlow and Gobius, ٢٠٠٦; Marquez et al., ٢٠٠٨]. Class ٢-like integrases have been found to be abundant in sediments with high input of sewage and fecal wastes, while poorly represented in the environments with moderate or no recent anthropogenic impact [Minguela-Rodriguez et al., ٢٠٠٩]. The presence of *intI٢*-like integrase in environments impacted by fecal waste from animals, including humans, allows us to suggest that *intI٢* may have been extracted from the extant environmental pool, presumably through the food chain and in the absence of antibiotic selection, and then laterally transferred and enriched in the gastrointestinal tract. These events seem feasible, as the ability of exogenous bacteria to survive transit in the human and animal gut environment and the occurrence of bacterial lateral gene transfer in the marine setting are well documented [Shoemaker et al., ٢٠٠١; Minguela-Rodriguez et al., ٢٠٠٩]. Even less is known about the class ٣ variants, which so far have been described in only three clinical instances [Xu et al., ٢٠٠٧].

Recent studies on marine integron metagenomes recorded high diverse integrases that have unique phylogenetic lineages (**Fig. ٣**), expanding the diversity of *intI* beyond the three traditional classes. More than ٢٨٠ *intI* phylotypes were recorded in metagenomes isolated from varieties of marine environments including hydrothermal vent fluids, symbionts and marine sediments from Arctic [Elsaied et al., ٢٠٠٧; Minguela-Rodriguez et al., ٢٠٠٩], a fact concerning the wide biogeographic distribution of integron in marine environments. The most interesting

feature of these *intI* diversities is the phylogenetic distinction of *intI* phylotypes between the studied environments. This may refer to the wide geographic distance and topology between the studied marine habitats.

Despite the large diversity of the recorded *intI*, the expectation of real diversity of *intI* in the studied marine environments has no upper limit as most of the studied *intI* clone libraries have yet to be saturated. More *intI* monitoring and improving techniques like developing new efficient *intI* PCR primers and qPCR will expand our knowledge about diversity of *intI* in marine environments.

٤. How big is the gene cassette pool in marine environment?

Culture-independent PCR/sequencing approaches yielded more than ٣٠٠٠ gene cassettes from different marine integron metagenomes including ٢١٤٥, ٧٠٨, ١٦٧ and ٤٦ cassettes from oil and sewage polluted marine sediments, coal and steel contaminated shores, deep-sea and heavy metals polluted estuaries, respectively, (**Fig. ٤**) [Elsaied et al., ٢٠٠٧, ٢٠١١, ٢٠١٣; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨]. However, these numbers of gene cassettes represent a tiny fraction of real gene cassette pool in each metagenome. This expectation supported by rarefaction analyses, based on gene cassette clone libraries, which indicated that sampling has yet to capture the diversity found in clone libraries from these sampling sites [Elsaied et al., ٢٠١١, ٢٠١٣; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨]. It should be considered that cassette analyses were done from relatively few samples, making these studies a substantial underestimate of marine integron gene cassette metagenome diversity. Primers used in these studies were designed from nucleotide alignments of a relatively small subset of *attC* sites [Stokes et al., ٢٠٠١]. However, the recorded *attC* sites are highly variable, demonstrating that the level of diversity of these *attC* elements continues to grow with cassette richness. Moreover, in all

studies done on marine integron gene cassette metagenomes, the used PCR approach was not sufficient to pick the full length of integrated gene cassettes, where the sizes of PCR ampilicons were short, consisted of, mostly, a single gene cassette [Elsaied et al., ٢٠١١, ٢٠١٣; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨].

Most of the recorded gene cassettes contained ORFs oriented in forward expression direction. However, some gene cassettes, with no apparent ORFs and contained only *attC*, were recorded in marine integrons. It is certain that more exhaustive sampling and additional methodology, in terms of gene cassette data collection, will reveal more cassette diversity in the marine environments.

٥. Marine integron gene cassette metagenome is a big reservoir of known and unknown gene cassette-coding proteins

The integrated gene cassettes recorded in marine integron metagenomes have structural features such as shortage of their containing ORFs (from ٤٥٠ bp to ١.٥ kb), mostly having ribosomal binding site upstream to the ORF and ORF initial codons ATG, GTG or TTG [Elsaied et al., ٢٠٠٧; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨]. These features facilitate easy expression of cassette ORFs, using integron cassette promoter, *Pc*, into proteins, which involve in different bacterial activities.

Most of gene cassette ORFs from clinical field encode antibiotic resistance proteins. Given that the lateral gene transfer plays an efficient role in moving the integron metagenome from clinical to urban marine environments, antibiotic resistance genes from clinical isolates have yet to be recorded in marine sediments. This is may be due to the limitation of gene cassette survey in marine environment. Other explanation is that integron may use its unique feature to disseminate antibiotic resistance genes, when the bacterium moves from clinic to marine

environment, where there is no stress of antibiotics. However, more studies are needed to confirm this concept.

Blast homology search showed that gene cassette ORFs, picked from marine integron metagenomes, encoded several categories of proteins (**Fig. ٥**). The first category included environment-adaptive proteins, which may be expressed by integron under exposure of bacterium to surrounding environmental selective pressures. Xenobiotic-degrading enzymes and pollutant signal proteins are the most recorded marine environment adaptive cassette proteins (Koenig et al., ٢٠٠٩; Elsaied et al., ٢٠١١). Protein bioinformatics predicted cell membrane localization of the cassette signal proteins, an implication for their functions as biosensors to surrounding environmental stresses. Some cassette adaptive proteins were expressed as a response of specific environment selective pressures, such as heat shock chaperon cassette proteins that may play a role in protection of bacteria from high temperature of deep-sea hydrothermal vents [Elsaied et al., ٢٠٠٧]. Several gene cassettes encoded catechol ١,٤-dioxygenase (EC ١.١٣.١١.١) and ٤-hydroxy-٢-oxovalerate aldolase (EC ٤.١.٣.٣٩), enzymes involve in degradation of aromatic industrial wastes such as benzoate, toluene and xylene, were recorded in coal and steel industrial waste contaminated marine sediment, an implication for involving of cassette proteins in the field of bioremediation [Koenig et al., ٢٠٠٩].

Other category consisted of cassette metabolism-related proteins. The most obvious examples of these proteins were transferase enzymes and potassium and iron ion transporter proteins, which were recorded in almost all studied inetgron metagenomes, suggesting the wide biogeographic distribution of these mobile cassette proteins in marine environments [Elsaied et al., ٢٠٠٧, ٢٠١١, ٢٠١٣; Koenig et al., ٢٠٠٨, ٢٠٠٩; Wright et al., ٢٠٠٨]. In addition, deep-sea integron metagenomes were found to be rich with cassette ORFs encoding specific enzymes such

as glycoside hydrolase, malate dehydrogenase and thioredoxin reductase beside DNA-binding enzymes such as transposases, suggesting the role of transposon as integron metagenome carrier in deep marine environment [Elsaied et al., ٢٠٠٧].

Cassette-encoding structural proteins constituted other category in marine gene cassette metagenomic pool. Some cassettes encoded novel protein domains and families such as a new family of nuclease-related domain (NERD) proteins, which were found in a broad range of bacterial, as well as single archaeal and plant proteins. The presence of NERD in the virulence-related pXO١ plasmid of *Bacillus anthracis* as well as in several other pathogens makes it a possible drug target [Grynberg and Godzik, ٢٠٠٤; Elsaied et al., ٢٠٠٧].

We have found examples of the IS٤ family of transposons linked to integrons from deep biosphere [Elsaied et al., ٢٠٠٧, ٢٠١١, ٢٠١٣]. Because the deep-sea microbial ecosystem has been considered as a primitive fraction of the biosphere, the transposon, IS٤ family, might be responsible for horizontal transfer of integrons and associated cassette genes in early history. However, this hypothesis would require further study.

The last category included cassette proteins, which have unknown functions and constituted the major fraction of cassette proteins recorded in marine environments. These unknown cassette proteins need characterization based on both protein prediction bioinformatics and experimental analyses. Thus, marine integron gene cassette metagenome has been considered as a source of unlimited number of mobile genes that encode novel proteins, which may have importance in the terms of scientific and commercial values.

٦. Integron metagenome as an active player in deep marine bacterial adaptation, the case is a deep-sea symbiont

The example of utilizing adaptive gene cassette metagenome in deep marine environment was represented by a deep-sea hydrothermal vent mussel sulfur-oxidizing endosymbiont. This endosymbiont lives inside the gill cells of the host mussel [Cavanaugh, ١٩٨٣] (**Fig. ٦a**). Hence, this symbiont is continuously exposing to various surrounding vent selective pressures. The endosymbiont utilizes hydrogen sulfide, erupted from the hot vent, as an electron donor for assimilation of organic carbon through the process of chemosynthesis [Felbeck et al., ١٩٨١].

Three groups of integron gene cassettes have been studied in this symbiont and encoded proteins with functions adapted to the symbiont life within the host animal [Elsaied et al., ٢٠٠٧]. Expression studies were performed on these gene cassettes in order to confirm their protein products and functions (**Fig. ٦b**) [Elsaied et al., unpublished data]. The first group was found to encode methionine aminopeptidase. Methionine is a sulfur-containing essential amino acid and is used as a source of sulfur in the animal symbiont-containing tissue [Duplessis et al., ٢٠٠٤]. Specifically, methionine aminopeptidase helps in liberating sulfur, from methionine, which is utilized as an electron sink, instead of oxygen, to produce sulfide during anoxic condition [Duplessis et al., ٢٠٠٤]. Sulfide production in gut flora from the large intestine in humans has been attributed to this mechanism [Magee et al., ٢٠٠٠]. Other gene cassette proteins matched with aminopeptidase N and O-sialoglycoprotein endopeptidase, enzymes playing a role in the fermentation of amino acids, which have been used, under certain conditions, as a source of carbon and energy in several deep-sea hydrothermal vent bacteria [Hou et al., ٢٠٠٤].

The second group of symbiont gene cassettes encoded glutamate synthase. This enzyme is a complex iron-sulfur flavoprotein that catalyses the reductive transfer of the amido nitrogen from L-glutamine to ٢-oxoglutarate to form two molecules of L-glutamate, a reaction in the

symbiont metabolic pathway for ammonia assimilation [Lee and Childress, ١٩٩٤; Minic and Herve, ٢٠٠٤].

The third group included gene cassette proteins matched with DNA repair proteins. Genomes are subjected to damage by physical and toxic chemical agents in the deep-sea hydrothermal vent environment. For example, increased temperature at vent sites is known to have the potential to cause DNA damage [Pruski and Dixon, ٢٠٠٣]. Another possible cause of DNA damage is the radioactive materials, which always occur at high levels in hydrothermal habitats [Cherry et al., ١٩٩٢]. As the endosymbiont is located in the external gill organ of the mussel, it is always directly exposed to these harmful conditions. Hence, DNA repair proteins have been recorded in vent mussel symbionts [Pruski and Dixon, ٢٠٠٣]. Generally, the presence of these mobile gene cassettes suggested that their associated proteins may be adaptive and used as a community resource in this specialized symbiont environment.

٧. Problems and prospective

Only, very few studies concerning integron gene cassette metagenome in marine environments have been reported. There is still lack information about the complete structure of marine integron metagenome. All previously used PCR primers pick only partial sequences of integrase genes or maximum three gene cassettes from long expected gene cassette arrays. In addition to these methodological limitations, most of the recorded gene cassettes encoded proteins with no significant homology with those in databases and new methodologies must be developed to uncover the functions of these cassette proteins.

However, this review gave background about the discovery of integron metagenome in marine environment and highlighted the importance of this mobile metagenome in molecular adaptation of marine bacteria. Future studies will focus on two aspects. The first aspect should include improvement the monitoring of integron metagenome in wide range of environments, supported with new concepts and methodologies. This will help in understanding the role of this mobile metagenome in environmental microbial ecosystem dynamics and function.

The second aspect will benefit from the function of integron, in collecting of environment adaptive functional genes, in developing synthetic integron recombinant system. This innovative system will help in screening adaptive genes useful for both of medical and industrial applications.

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