Integron associated mobile genes
Just a collection of plug in apps or essential components of cell network hardware?

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Lateral gene transfer (LGT) impacts on the evolution of prokaryotes in both the short and long-term. The short-term impacts of mobilized genes are a concern to humans since LGT explains the global rise of multi drug resistant pathogens seen in the past 70 years. However, LGT has been a feature of prokaryotes from the earliest days of their existence and the concept of a bifurcating tree of life is not entirely applicable to prokaryotes since most genes in extant prokaryotic genomes have probably been acquired from other lineages. Successful transfer and maintenance of a gene in a new host is understandable if it acts independently of cell networks and confers an advantage. Antibiotic resistance provides an example of this whereby a gene can be advantageous in virtually any cell across broad species backgrounds. In a longer evolutionary context however laterally transferred genes can be assimilated into even essential cell networks. How this happens is not well understood and we discuss recent work that identifies a mobile gene, unique to a cell lineage, which is detrimental to the cell when lost. We also present some additional data and believe our emerging model will be helpful in understanding how mobile genes integrate into cell networks.

Lateral Gene Transfer and Bacterial Evolution

Lateral gene transfer (LGT) is a phenomenon central to prokaryotic evolution and adaptation. With the increasing use and sophistication of genomics technologies and bioinformatics tools, estimates of the extent of laterally transferred genes have tended upwards. A recent study estimated that an average of 81% of the genes in any given prokaryote had been transferred at some point in their history.[1] The existence and extent of LGT has long been a vexing issue in biology. In respect of microbiology it has led to a long debate of the species concept as it applies to prokaryotes.[2] In the broader evolutionary debate LGT has also challenged aspects of neo Darwinism especially in regards to the notion of evolution by gradual change.[3]

While all microbiologists accept LGT as a fact, there are still difficulties in reconciling the observation of its large contribution to genomes in the long-term and the likely fitness impact on a genome of acquiring one or more gene units of DNA in the short-term.[3,4] With as little as 20% of a bacterial genome being comprised of genes that respect the classical evolutionary laws of inheritance by vertical descent it follows that most laterally acquired genes must be integrated into essential interconnected metabolic or regulatory pathways (cell networks). Neo-Darwinism argues that evolution of genes integrated into cell networks occurs by mutational changes in existing genes or duplicated genes either of which randomly generates subtle fitness advantages. Over time these can lead to networks with distinct properties in different species and, indeed, facilitate the process of speciation itself. It can also lead to the generation of completely new pathways and networks. In contrast, the sudden introduction of a new gene or genes into a bacterium is an "instantaneous" change. In contrast to a subtle point mutation in an existing or duplicated gene, this is much less likely to provide an opportunity for the new gene to co evolve with, and adapt to, existing cell networks. In the absence of other changes this would mean the transferred gene would not become established in a population. A partial explanation for this dilemma may be in the finding that mobile DNA might be silenced by host defenses preventing detrimental short-term effects.[5]

Putting aside the difficulties of integrating an instantaneously acquired innovation into a cellular network, it is universally understood that some types of laterally transferred genes can have enormous positive fitness impacts on a bacterial cell. An obvious example is antibiotic resistance. With the clinical introduction of antibiotics in the mid 20th century, it was not long before resistant bacteria began to emerge. The rapid emergence and spread of antibiotic resistance was one of the earliest indicators of evolution by LGT. Today, the ubiquitous presence of multi drug resistant bacteria has led

the World Health Organization to recognize the resulting decline in antibiotic efficacy as one of the great health challenges of the 21st century. Most identified antibiotic resistance genes are part of the mobile genome and the selection for antibiotic resistance has facilitated the assembly and concentration of the genes conferring resistance into a plethora of mobilizing elements.[6] This enormous resistance gene Diaspora can be attributed to strong artificial selection by human activities and is very recent. The presence of these laterally transferred genes in diverse bacteria is relatively easy to understand. First, selection for resistance is very strong, at least in some environments. Thus, in many contexts, the fitness of cells lacking resistance genes is essentially zero. Also, resistant bacteria are predicted to persist in the environment even if all uses of antibiotics ceased.[7] The reasons for persistence in the absence of selection are not all entirely clear although one explanation for the reduced fitness cost is that most acquired resistance genes or gene pathways act autonomously and outside cell metabolic networks. This in itself helps to account for the rapid dissemination of genes through diverse pathogens since a resistance gene will work in any cellular context. Thus, the same gene can be found to be mediating resistance in many disparate pathogens.

Integration of Acquired Genes into Cell Networks

Prior to the genomic era, the bulk of our understanding of LGT was drawn from highly mobile genes and the elements that mobilized them. LGT was then considered a marginal process which did not concern most genes, including those used to reconstruct a phylogenetic Universal Tree of Life. The compatibility of our understanding of prokaryotic evolution to neo-Darwinian concepts was not questioned, as LGT was only viewed to affect a minority of specialized genes. The microbial genomics era, which began in 1995, with every passing year and additional sequenced genome, progressively led to the incontrovertible conclusion that LGT has been a dominant force in microbial

evolution.[8] Thus, rather than just adding on genes in response to strong selection that act independent of the cell network, laterally transferred genes were key integrated components in the cell.

Several recent studies have shown that phylogenies of gene families that are involved in cell networks do not conform to a simple bifurcating tree type model as would be expected for vertical inheritance.[1,9,10] The likely extent of LGT for such networked genes is still unclear. Pal et al.[9] have argued that LGT has played an important role in building up complex networks via involvement in pathways that allow bacteria to niche adapt to specialized environment types. Thus, an influx of genes at the periphery is most likely to assist bacteria in adapting to new environments and not by optimization in fixed environments. This model is attractive in that it is consistent with the diverse ecological niches that prokaryotes have come to inhabit. By analogy with the complexity hypothesis first advanced by Jain et al.,[11] this model predicts that metabolic genes rather than core informational genes are more likely to show evidence of LGT. There is clear evidence that this is true in a relative sense, however it is also the case that some informational genes also display evidence of frequent LGT. Even if mostly added at the periphery, it is clear that newly acquired genes need to be able to "communicate" with existing cell networks to some extent even if only, for example, to channel partially processed substrates into central metabolic networks. How this is achieved in the first instance will impact on fitness. It has thus been argued that the evolution of new integrated pathways requires the evolution or acquisition of regulatory proteins as well as enzymatic ones.[10]

Understanding how networks are impacted by LGT is crucial to fully integrating this phenomenon in evolutionary theory. It also has relevance to more applied branches of science, as cellular networks are critical in the evolution of pathogenicity[12] and impact on the evolution of pathogenic bacteria.[13] Thus, although the genomics era has told us a lot about the extent of LGT and where it has occurred, as pointed out by Davids

and Zhang, plausible mechanisms as to the events that lead to integration of acquired genes are lacking.[14]

The Integron/Gene Cassette System

For several years our laboratories have been investigating the biology of the integron/gene cassette system.[15] This system is an important component of the mobile genome in Gram-negative bacteria. It was first characterized in the context of its role in spreading antibiotic resistance genes in human pathogens.[16] In that regard, it is an exemplar of the power of the adaptive potential of mobile DNA since, although it was selected for and spread as a result of the heavy use of antibiotics by humans, the integron is an ancient structure that has been a feature of many bacterial genomes for a long period of evolutionary time.[17] The defining feature of all integrons is their ability to capture genes when the latter are part of mobilizable elements known as gene cassettes.[18] What makes integrons arguably unique in the plethora of mobile elements is that they appear to be highly adapted as a tool kit for natural experimental evolution. Their key feature is their ability to insert any gene cassette at a defined integron associated recombination site by site-specific recombination. The advantage of this process is that it allows insertion of a DNA sequence at a defined location in the genome which otherwise does not disrupt any other gene in the cell by insertional inactivation. It also allows the immediate expression of the newly acquired gene, as a promoter is located next to the insertion site. Unlike other site specific recombination systems however, insertion does not involve a single discrete DNA sequence such as a lysogenic phage but rather, a diversity of sequences most of which include defined genes, that to date has no definable upper limit in terms of numbers.[19,20] Thus mobilized genes acquired by LGT can be inserted and expressed in a way that does not otherwise impact on cellular gene content. Other evidence that this system is designed to facilitate adaptive innovation is the fact that the site-specific recombination reaction is genetically regulated such that the SOS response leads to an

increase in mobile cassette rearrangement frequencies.[21] Therefore, this provides a mechanism for generating diversity at times when cells have to rapidly adjust to new or changing environments.

Apart from the large number of mobile genes known to exist, another defining feature of the cassette metagenome is the extraordinary amount of novelty it contains.[22] This novelty extends to the point that most genes found within the cassette metagenome either possess no identified homologs or are homologous to genes encoding proteins that are identified only as "conserved hypotheticals" in databases. One of the great challenges in this area of research is in understanding what these mobile genes do, especially given the fact that they, collectively, comprise a resource that must be many orders of magnitude larger than any single bacterial genome. In our view, however, there is no question that the vast majority of cassette-encoded proteins are adaptive. For example, structural biology approaches (by obtaining high resolution crystal structures) have revealed functions for many such proteins. Thus, we have found via these approaches that some cassette proteins include putative house cleaning functions[23] and ligand binding domains commonly associated with two component transcriptional regulators[24] both of which are likely to impact on cell networks. This latter example is particularly interesting as it implies that the modular rearrangement of protein domains via cassette shuffling may be a precursor for the evolution of multi domain proteins. One could envisage a scenario whereby two adjacent cassettes providing complementary functions may become fused by loss of the cassette recombination site creating a new multi domain protein. Cassette fusion has been previously observed.[25] In any event, the notion that mobile cassettes may encode transcription regulators as well as enzymatic proteins is consistent with this genetic element constituting an adaptive tool-box.[10] Apart from potentially evolving new proteins, cassette uptake in natural environments[26] and shuffling may be a process for operon creation by bringing together functionally distinct proteins that can cooperate to form a co-regulated biochemical pathway.[27]

Lineage-Specific Integration of a Recently Acquired Gene into an Essential Cell Network

Other approaches besides structural biology have been used to understand what role cassette-encoded proteins play in the cell. Our major model for this is the *Vibrio rotiferanus* strain DAT722, the genome of which we have recently sequenced.[28] From an integron perspective, this species is typical of the vibrios in that it has large cassette arrays[29]—composed of 116 cassettes in the case of DAT722—with most of the associated proteins having no identifiable function. This bacterium is also both nonpathogenic to humans and amenable to genetic manipulation. This makes it and its close relatives useful models for testing of specific hypotheses.

In a recent study we examined the impact of cassette array deletions on the metabolic capacity of the DAT722 cells.[30] This was initially done by examining the ability of the mutants to grow on a variety of carbon sources in a Biolog screening assay. Our intent was to try and identify mutants with a varied capacity to metabolize specific substrates. Surprisingly, we found that some mutants concomitantly had a greatly reduced viability in minimal media in the presence of a number of different carbon substrates including glucose. In contrast, mutant growth was normal in rich media. Furthermore, these mutants exhibited a hypermutative phenotype (Labbate M., unpublished) indicating the deletion had resulted in the loss of a significant gene which made the bacterium maladapted to its environment. This change of phenotype was ascribed to one specific protein, encoded within cassette 11 (the 11th cassette in the 116 cassette array). The cassette 11 protein was demonstrated to have a role in porin regulation and the altered growth profiles were a consequence of changes in porins. The reduction in fitness on deletion of cassette 11 is substantial to the point of making the cell nearly non viable in certain carbon-containing minimal growth media. To our knowledge this is the first experimental data that demonstrates the integration of an apparently unique mobile gene into an important cell network. One particularly interesting aspect to this is the

fact that the negative impact on growth is specific for a media that closely resembles the environment in which free living vibrios are most commonly found—namely estuarine water.

Results

To test whether cassette 11 protein can impact on the fitness of other *Vibrio* strains, we introduced the cassette 11 gene containing recombinant vector pMAQ1082[30] into a *V. cholerae* strain designated S25 to determine whether fitness was affected. pMAQ1082 comprises the cloning vector pJAK16 into which the cassette 11 gene has been cloned under the control of an IPTG inducible promoter. S25 is an environmental nonO1/nonO139 *V. cholerae* strain isolated from a Sydney, Australia, estuarine environment. It has a large integron array but does not possess cassette 11 or a close homolog based on PCR using primers targeting this cassette. S25 with and without cassette 11 had identical growth characteristics (**Fig. 1**) in complete media a result identical to that for DAT722 with and without this cassette.[30] In contrast to DAT722 however, which had greatly altered growth rates in most minimal media, including 2M + glucose, when isogenic strains with and without cassette 11 were compared,[30] the growth of S25 in the same media was unaffected by the presence or absence of this cassette and was identical to the growth seen in complete media (**Fig. 1**). The simplest interpretation of this is that the cassette 11 protein does not interact with any S25 cell networks in stark contrast to its influence on networks in DAT722. While more data are needed, we speculate that integration of some mobile genes into cell networks may be analogous to the evolution of duplicated genes in eukaryotes. In eukaryotes duplication is most commonly via the generation of identical copies of an existing gene. In contrast, the introduction of cassette 11 into the ancestor of DAT722 initially may have had no impact on the cell as this progenitor possessed a non identical gene that nonetheless encoded a protein with a related function. As this strain evolved however incremental changes led to cassette 11 protein replacing this pre
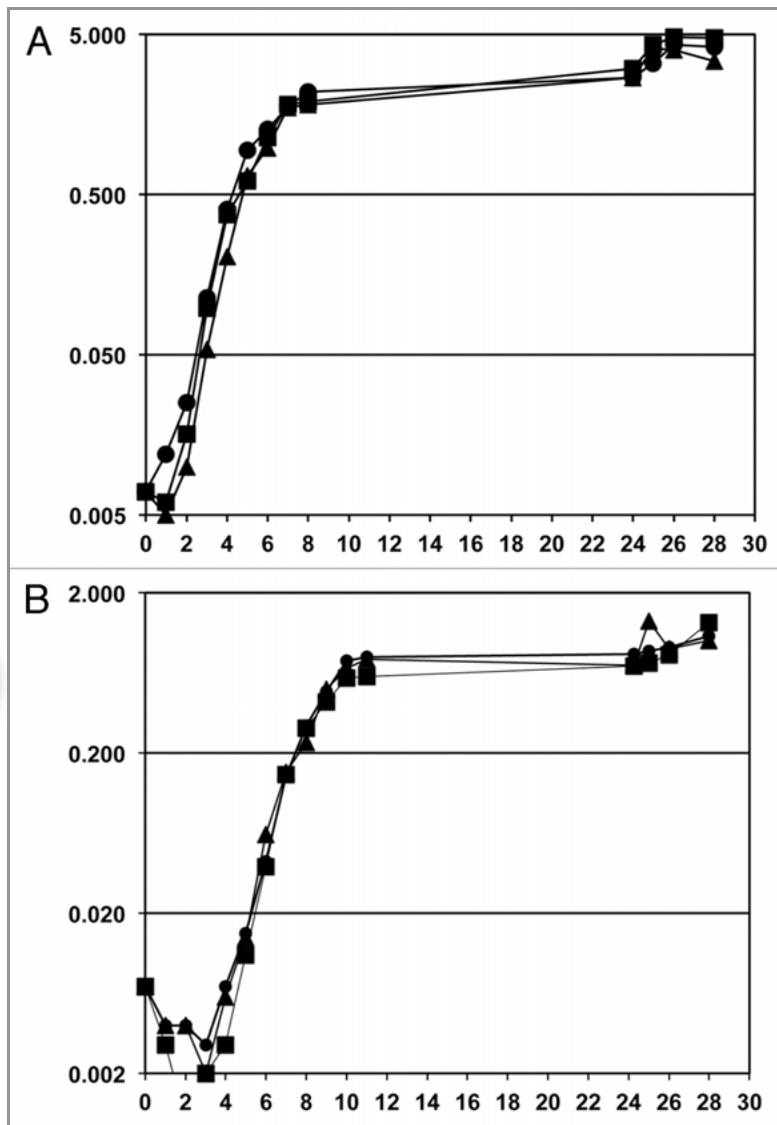
Figure 1. Impact of cassette 11 protein in *V. cholerae* S25. Growth curves of *V. cholerae* S25 (squares), *V. cholerae* S25/pJAK16 (circles) and *V. cholerae* S25/pMAQ1082 (triangles) induced with 0.1 mM IPTG in LB20 (A) and 2M + glucose (B).

existing protein in terms of its central network role. *V. cholerae* S25 (pMAQ1082) may be a useful experimental evolution model for exploring this hypothesis.

What is the cassette 11 encoded protein? At this time we do not have a definitive answer although its main target is most likely DNA and consequently it may play a role in regulation of DNA supercoiling. This potential link is inferred by the presence of two distinct domains in the protein. One of these is a C-terminal zinc finger domain commonly associated with prokaryotic DNA topoisomerase I proteins and in these proteins catalyzes the relaxation of supercoiled DNA. The second is a recently identified nuclease related NERD domain[31] inferred to have a role in DNA processing. We have found this domain is present in proteins found in diverse bacteria (**Fig. 2**), a distribution suggestive of spread by LGT as is the case for the cassette 11 protein family overall. Interestingly this bioinformatic analysis reveals examples of this NERD domain being encoded by genes in highly

mobilized elements (gene cassettes and transposons) as well as being fixed in cell lines for (presumably) longer periods of time as a result of the gene being located on a chromosome. Obtaining a crystal structure and identifying a precise biochemical function of the cassette 11 protein would be of great interest. An implied role in DNA processing is tantalizing as this type of information processing function is one of the least likely candidates for successful LGT according to the complexity hypothesis or the network evolution model advanced by Pal.[9] Understanding its precise role is likely to shed light on the forces that allow and provide for rapid integration of LGT derived genes into cell networks. It could also bring insights into speciation processes. If a gene can be integrated into a cell line specific network such that its loss is nearly fatal, this integration event may represent the first step in a process that represents sympatric speciation.

Conclusions

In summary, core prokaryotic genes defined by little identifiable evidence of LGT over long evolutionary periods constitute only a small minority of the genes in extant genomes. The majority of genes have at some point been acquired by LGT, including those that are now fixed on chromosomes and integrated into the cell networks that support prokaryotic life. It is these genes that have allowed niche specialization and adaptation of bacteria to novel environments. A complete understanding of the process of LGT requires an understanding of how new genes integrate into cell networks. This Systems Biology understanding will require hypothesis driven experimental approaches as well as those involving genomics and bioinformatics.
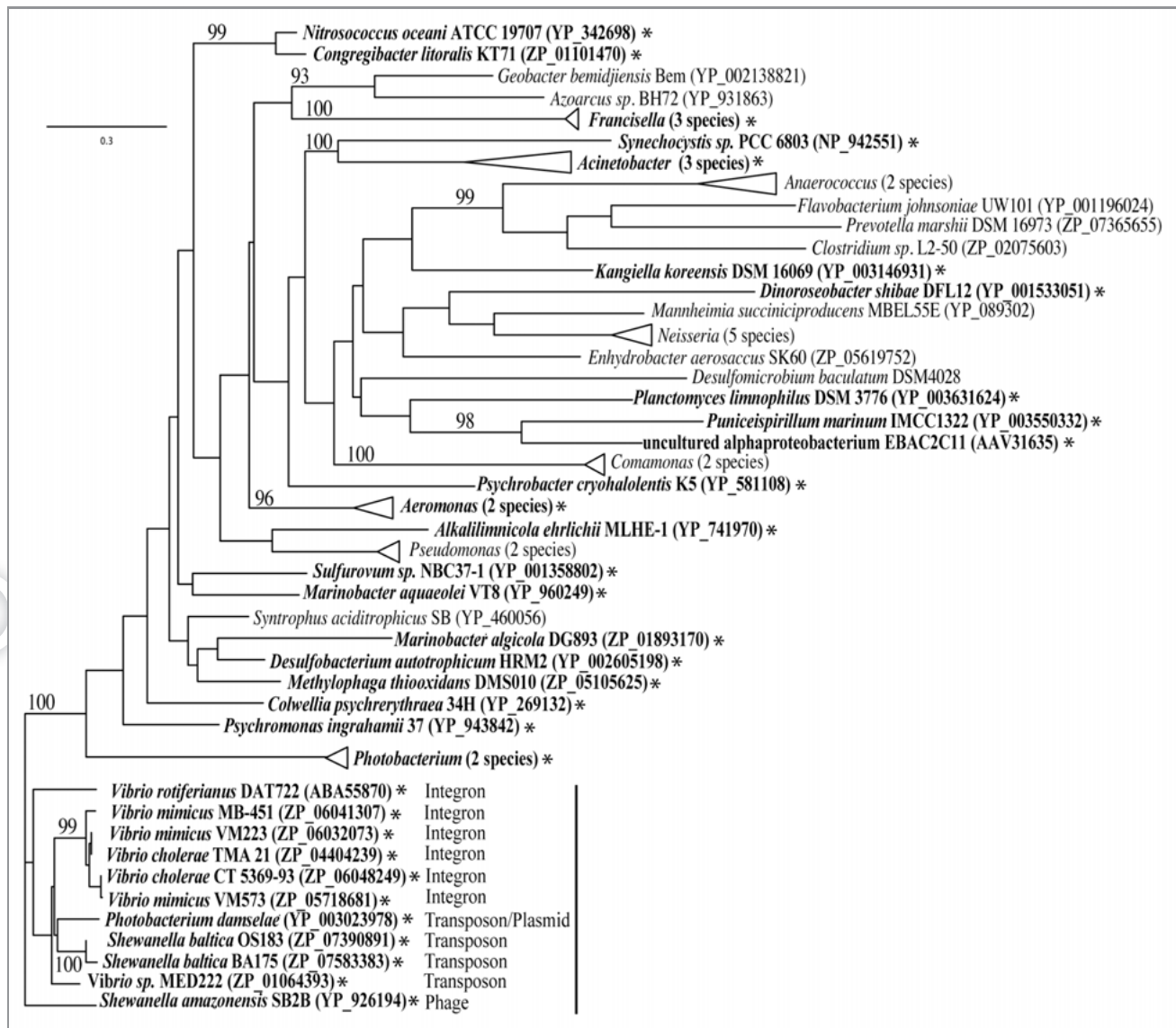
Figure 2. Phylogeny of NERD proteins similar to cassette 11 protein from *V. rotiferianus* DAT722. Each taxon name is followed by the accession number of the protein it contains, as well as the genetic element the protein is associated with. The phylogeny has been reconstructed by maximum likelihood using RAxML. Confidence values over 80% are displayed on nodes of interest, representing the proportion of bootstrap pseudo-replicates supporting topology. Proteins containing a C4-zinc finger domain are in bold. An asterisk indicates marine/aquatic bacteria. The line marks the clade containing cassette 11 protein.

References

1. Dagan T, Artzy-Randrup Y, Martin W. Modular networks and cumulative impact of lateral transfer in prokaryote genome evolution. Proc Natl Acad Sci U S A 2008; 105:10039-44; PMID:18632554; http://dx.doi.org/10.1073/pnas.0800679105

2. Riley MA, Lizotte-Waniewski M. Population genomics and the bacterial species concept. Methods Mol Biol 2009; 532:367-77; PMID:19271196; http://dx.doi.org/10.1007/978-1-60327-853-9_21

3. Boto L. Horizontal gene transfer in evolution: facts and challenges. Proc Biol Sci 2010; 277:819-27; PMID:19864285; http://dx.doi.org/10.1098/rspb.2009.1679

4. O'Malley MA, Boucher Y. Paradigm change in evolutionary microbiology. Stud Hist Philos Biol Biomed Sci 2005; 36:183-208; PMID:16120264; http://dx.doi.org/10.1016/j.shpsc.2004.12.002

5. Stoebel DM, Free A, Dorman CJ. Anti-silencing: overcoming H-NS-mediated repression of transcription in Gram-negative enteric bacteria. Microbiology 2008; 154:2533-45; PMID:18757787; http://dx.doi.org/10.1099/mic.0.2008/020693-0

6. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol Rev 2011; 35:790-819; PMID:21517914; http://dx.doi.org/10.1111/j.1574-6976.2011.00273.x

7. Andersson DI, Hughes D. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev 2011; 35:901-11; PMID:21707669; http://dx.doi.org/10.1111/j.1574-6976.2011.00289.x

8. Koonin EV, Wolf YI. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. Nucleic Acids Res 2008; 36:6688-719; PMID:18948295; http://dx.doi.org/10.1093/nar/gkn668

9. Pál C, Papp B, Lercher MJ. Adaptive evolution of bacterial metabolic networks by horizontal gene transfer. Nat Genet 2005; 37:1372-5; PMID:16311593; http://dx.doi.org/10.1038/ng1686

10. Maslov S, Krishna S, Pang TY, Sneppen K. Toolbox model of evolution of prokaryotic metabolic networks and their regulation. Proc Natl Acad Sci U S A 2009; 106:9743-8; PMID:19482938; http://dx.doi.org/10.1073/pnas.0903206106

11. Jain R, Rivera MC, Lake JA. Horizontal gene transfer among genomes: the complexity hypothesis. Proc Natl Acad Sci U S A 1999; 96:3801-6; PMID:10097118; http://dx.doi.org/10.1073/pnas.96.7.3801

12. Jackson RW, Vinatzer B, Arnold DL, Dorus S, Murillo J. The influence of the accessory genome on bacterial pathogen evolution. Mob Genet Elements 2011; 1:55-65; PMID:22016845; http://dx.doi.org/10.4161/mge.1.1.16432

13. Dillon SC, Cameron AD, Hokamp K, Lucchini S, Hinton JC, Dorman CJ. Genome-wide analysis of the H-NS and Sfh regulatory networks in *Salmonella Typhimurium* identifies a plasmid-encoded transcription silencing mechanism. Mol Microbiol 2010; 76:1250-65; PMID:20444106; http://dx.doi.org/10.1111/j.1365-2958.2010.07173.x

14. Davids W, Zhang Z. The impact of horizontal gene transfer in shaping operons and protein interaction networks–direct evidence of preferential attachment. BMC Evol Biol 2008; 8:23; PMID:18218112; http://dx.doi.org/10.1186/1471-2148-8-23

15. Roy Chowdhury P, Stokes HW, Labbate M. Integrons. Antibiotic resistance and beyond. In: Bacterial Integrative Mobile Genetic Elements. Edited by Roberts AP; 2012.

16. Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M, et al. The evolution of class 1 integrons and the rise of antibiotic resistance. J Bacteriol 2008; 190:5095-100; PMID:18487337; http://dx.doi.org/10.1128/JB.00152-08

17. Cambray G, Guerout AM, Mazel D. Integrons. Annu Rev Genet 2010; 44:141-66; PMID:20707672; http://dx.doi.org/10.1146/annurev-genet-102209-163504

18. Recchia GD, Hall RM. Origins of the mobile gene cassettes found in integrons. Trends Microbiol 1997; 5:389-94; PMID:9351174; http://dx.doi.org/10.1016/S0966-842X(97)01123-2

19. Holmes AJ, Gillings MR, Nield BS, Mabbutt BC, Nevalainen KM, Stokes HW. The gene cassette metagenome is a basic resource for bacterial genome evolution. Environ Microbiol 2003; 5:383-94; PMID:12713464; http://dx.doi.org/10.1046/j.1462-2920.2003.00429.x

20. Michael CA, Gillings MR, Holmes AJ, Hughes L, Andrew NR, Holley MP, et al. Mobile gene cassettes: a fundamental resource for bacterial evolution. Am Nat 2004; 164:1-12; PMID:15266366; http://dx.doi.org/10.1086/421733

21. Guerin E, Cambray G, Sanchez-Alberola N, Campoy S, Erill I, Da Re S, et al. The SOS response controls integron recombination. Science 2009; 324:1034; PMID:19460999; http://dx.doi.org/10.1126/science.1172914

22. Boucher Y, Labbate M, Koenig JE, Stokes HW. Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends Microbiol 2007; 15:301-9; PMID:17566739; http://dx.doi.org/10.1016/j.tim.2007.05.004

23. Robinson A, Guilfoyle AP, Harrop SJ, Boucher Y, Stokes HW, Curmi PM, et al. A putative house-cleaning enzyme encoded within an integron array: 1.8 A crystal structure defines a new MazG subtype. Mol Microbiol 2007; 66:610-21; PMID:17892463; http://dx.doi.org/10.1111/j.1365-2958.2007.05932.x

24. Deshpande CN, Harrop SJ, Boucher Y, Hassan KA, Di Leo R, Xu X, et al. Crystal structure of an integron gene cassette-associated protein from *Vibrio cholerae* identifies a cationic drug-binding module. PLoS One 2011; 6:e16934; PMID:21390267; http://dx.doi.org/10.1371/journal.pone.0016934

25. Naas T, Mikami Y, Imai T, Poirel L, Nordmann P. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of *Escherichia coli* which carries an unusual array of gene cassettes. J Bacteriol 2001; 183:235-49; PMID:11114922; http://dx.doi.org/10.1128/JB.183.1.235-249.2001

26. Gestal AM, Liew EF, Coleman NV. Natural transformation with synthetic gene cassettes: new tools for integron research and biotechnology. Microbiology 2011; 157:3349-60; PMID:21948046; http://dx.doi.org/10.1099/mic.0.051623-0

27. Bikard D, Julié-Galau S, Cambray G, Mazel D. The synthetic integron: an *in vivo* genetic shuffling device. Nucleic Acids Res 2010; 38:e153; PMID:20534632; http://dx.doi.org/10.1093/nar/gkq511

28. Roy Chowdhury P, Boucher Y, Hassan KA, Paulsen IT, Stokes HW, Labbate M. Genome sequence of *Vibrio rotiferianus* strain DAT722. J Bacteriol 2011; 193:3381-2; PMID:21551292; http://dx.doi.org/10.1128/JB.05089-11

29. Rowe-Magnus DA, Guerout AM, Biskri L, Bouige P, Mazel D. Comparative analysis of superintegrons: engineering extensive genetic diversity in the Vibrionaceae. Genome Res 2003; 13:428-42; PMID:12618374; http://dx.doi.org/10.1101/gr.617103

30. Labbate M, Boucher Y, Roy Chowdhury P, Stokes HW. Integration of a laterally acquired gene into a cell network important for growth in a strain of *Vibrio rotiferianus*. BMC Microbiol 2011; 11:253; PMID:22093957; http://dx.doi.org/10.1186/1471-2180-11-253

31. Grynberg M, Godzik A. NERD: a DNA processing-related domain present in the anthrax virulence plasmid, pXO1. Trends Biochem Sci 2004; 29:106-10; PMID:15055202; http://dx.doi.org/10.1016/j.tibs.2004.01.002