PRINCIPLE OF BASIC MOLECULAR BACTERIOLOGY

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Organization

Kerman University of Medical Sciences

Identification of Pathogenic bacteria

1-Study of phylogenic relationship of CTXM gene among ESBL producing *Pseudomonas aeruginosa* by RFLP -sequencing.

2-Study on detection of MBL/ESBL β-lactamases in *Pseudomonas aeruginosa* isolated from burn patients in Kerman, Iran by PCR and multiplex -PCR.

Home | Project List | Employee List | Related Links

3-Study on mutations in exon 5 and exon 8 of tumor suppressor gene TP53 in sequamous lung cell carcinoma.

4-Study on Plasmids responsible for cefotaxim & ceftizoxime in nosocomial bacteria isolated from hospitals in Kerman, Iran.

5-tudy on curing activity of antibiotic resistance plasmids in *Klebsiella pneumonieae* using Iranian plant extracts.

6-Study on Antibiotic resistance, beta lactamase production and plasmid profile of *Neisseria gonorrheae* strains.

7-Study on antibiotic sensitivity of *pseudomonas aeruginosa* to ciprofloxacin isolated from burn patients.

8-Detection of *Legionella pneumophila* in Cooling Water Systems of Hospitals and Nursing Homes of Kerman City, Iran by Semi- Nested PCR

9- Study on Myrtus extracts and its anti-super coiling DNA (Co-worker).

10- Detection of *Neisseria gonorrhoeae* by multiplex PCR (Co-worker).

11- Horizontal transfer of antibiotic resistance genes among gram negative bacteria in sewage and lake water and influence of some physico-chemical parameters of water on conjugation process.

12- Study on isolation, species distribution, antibacterial resistance pattern and Beta-lactamase production of enterococci isolated from human samples in southeast of Iran.

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Table of contents

Bacterial Chromosomes

Introduction

In contrast to the linear chromosomes found in eukaryotic cells, the strains of bacteria initially studied were found to have single, covalently closed, circular chromosomes. The circularity of the bacterial chromosome was ele1gantly demonstrated by electron microscopy in both Gram negative bacteria such as *E. coli* and Gram positive bacteria such as *Bacillus subtilis*. Bacterial plasmids were also shown to be circular. In fact, the experiments were so beautiful and the evidence was so convincing that the idea that bacterial chromosomes are circular and eukaryotic chromosomes are linear was quickly accepted as a definitive distinction between prokaryotic and eukaryotic cells. However, like most other distinctions between prokaryotic and eukaryotic cells, it is now clear that this dichotomy is incorrect. Not all bacteria have a single circular chromosome: some bacteria have multiple circular chromosomes, and many bacteria have linear chromosomes and linear plasmids.

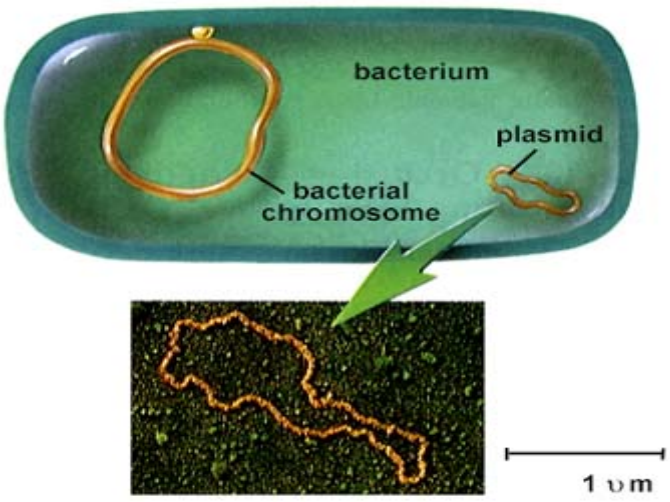


Figure1. Bacterial Chromosome

Experimental evidence for multiple chromosomes and linear chromosomes initially came from studies using pulsed field gel electrophoresis (PFGE), an approach that uses alternating electric fields to separate large DNA molecules on an agarose gel. Subsequently genome sequencing projects have added to the list of bacteria with multiple or linear chromosomes.

Some examples of bacterial genome organization

Bacteria	Chromosome(s)	Plasmid(s)
Agrobacterium tumefaciens	one linear (2.1 Mb) + one circular (3.0 Mb)	two circular (450 + 200 Kb)
Bacillus subtilis	one circular (4.2 Mb)	
Bacillus thuringiensis	one circular (5.7 Mb)	six (each >50 Kb)
Borrelia	one linear (0.91 Mb)	multiple circular + linear (5-200 Kb)
Bradyrhizobium japonicum	one circular (8.7 Mb)	
Brucella melitensis	two circular (2.1 + 1.2 Mb)	
Brucella suis biovars 1, 2, 4	two circular (1.0 + 2.0 Mb)	
Brucella suis biovar 3	one circular (3.1 Mb)	
Buchnera sp. strain APS	one circular (640 Kb)	two circular (< 7.8 Kb each)
Deinococcus radiodurans	two circular (2.6 + 0.4 Mb)	two circular (177 + 45 Kb)
Escherichia coli K-12	one circular (4.6 Mb)	
Leptospira interrogans	two circular (4.7 + 0.35 Mb)	
Paracoccus denitrificans	three circular (2.0 + 1.1 + 0.64 Mb)	
Pseudomonas aeruginosa	single circular (6.3 Mb)	
Rhizobacterium meliloti	two circular (3.4 + 1.7 Mb	one circular megaplasmid (1,400 Kb)
Rhodobacter sphaeroides	two circular (3.0 + 0.3 Mb)	
Ureaplasma urealyticum	one circular (0.75 Mb)	
Vibrio cholerae	two circular (2.9 + 1.1 Mb)	
Vibrio parahaemolyticus	two circular (3.2 + 1.9 Mb)	
Xylella fastidiosa	one circular (2.7 Mb)	two circular (51 + 1.3 Kb)

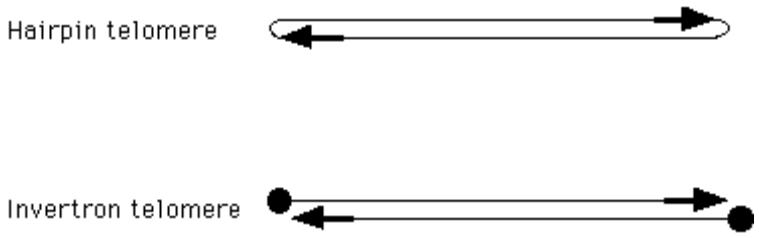
The first convincing evidence that some bacteria have multiple chromosomes came from studies on *Rhodobacter sphaeroides*. Both molecular and genetic studies clearly demonstrated that *R. sphaeroides* has two large circular chromosomes. One of the chromosomes is 3.0 Mb and the other is 0.9 Mb. Genes encoding rRNAs and tRNAs required for translation, and metabolic enzymes are distributed between the two chromosomes. Multiple chromosomes have also been found in many other bacteria, including *Agrobacterium tumefaciens, Rhizobium, Brucella, Paracoccus denitrificans,*

Ochrobactrum anthropi, Leptospira interrogans, Burkholderia, Vibrio cholerae, Deinococcus radiodurans, and many others from diverse groups of bacteria.

Furthermore, some bacteria have linear chromosomes. Borrelia have linear chromosomes and most strains contain both linear and circular plasmids; most of the bacteria in the genus Streptomyces have linear chromosomes and plasmids and some have circular plasmids as well. In addition, in some cases there may be a dynamic equilibrium between linear and circular forms of a DNA molecule. There is some evidence that linearization may be due to integration of a linear phage genome into the circular DNA molecule.

Linear chromosomes and plasmids were not discovered in bacteria until relatively recently. The first published evidence for linear chromosomes was in 1979, but because the techniques used at that time were limited[5] and because the dogma that all bacterial chromosomes are circular was so entrenched, few people believed that linear chromosomes and plasmids occurred in bacteria until 1989. By that time pulsed field gel electrophoresis had been developed, and this new technique provided convincing evidence that the chromosome of *Borrelia burgdoferi* was linear.

The ends of linear DNA molecules (called telomeres) pose two problems that do not apply to circular DNA molecules. First, since free double-stranded DNA ends are very sensitive to degradation by intracellular nucleases, there must be a mechanism to protect the ends. Second, the ends of linear DNA molecules must have a special mechanism for DNA replication. These problems are solved by features of the telomeres. Two different types of telomeres have been observed in bacteria: hairpin telomeres and invertron telomeres.



There are examples of linear DNA molecules in bacteria that are protected by both types of telomeres: palindromic hairpin loops are protected by the lack of free double-stranded ends, and invertron telomeres are protected by proteins that bind to the 5'-ends. Both of these mechanisms are also used by some phage, eukaryotic viruses, and eukaryotic plasmids. The two types of telomeres also solve the problem of DNA replication differently. Invertron telomeres have a protein covalently attached to the 5' ends of the DNA molecule (called the 5'-terminal protein or TP for short). DNA polymerase interacts with the TP at the telomere and catalyzes the formation of a covalent bond between the TP and a dNTP. The dNTP bound to the TP has a free 3'-OH group which acts as the primer for chain elongation. Replication of hairpin telomeres is less well understood. Apparently multiple hairpin sequences can pair to form concatemers that are replication intermediates.

The important take-home point is that we are just beginning to appreciate the similarity of many processes once thought to be completely different between bacteria and eukaryotes, partly because we now have better tools for studying these processes and partly because most of the earlier studies focused on relatively few types of bacteria. The more we study a wider diversity of bacteria, phages, and plasmids, the more obvious it becomes that *E. coli* is an excellent model for dissecting broad features of molecular and cell biology, but not all bacteria do everything the same way. Furthermore, we have only recently begun to attack the molecular genetics of the

Archae, and what we have learned so far suggests that this diverse group of prokaryotes share even more common features with the eukaryotes.

1. The circular genomes of mitochondrial and chloroplast are a notable exception to the rule that eukaryotic chromosomes are linear. However, this nicely fit into the dichotomy that eukaryotic chromosomes are linear and bacterial chromosomes are circular because these organelles seem to have evolved from entrapped bacteria.
2. Other examples include the presence of introns, and poly-A tails on mRNA.
3. This genus includes *B. burgdorferi*, the causative agent of Lyme disease.
4. Streptomyces make a wide variety of useful antibiotics, including streptomycin.
5. For example, linear DNA was precipitated in the most commonly used procedures for purifying bacterial plasmids, and the procedures for purifying chromosomal DNA relied upon the differential binding of ethidium bromide to "sheared DNA fragments" compared to circular DNA.
6. It is not intuitively obvious how the ends of a linear DNA molecule could be completely replicated. All known DNA polymerases require a pre-existing primer for initiation of DNA replication. The primer is usually a short RNA molecule with a free 3'-OH group that can be extended by DNA polymerase. If a linear DNA molecule was primed at one end, DNA synthesis could continue to the other end. However, once the primer is removed, the DNA corresponding to the primer could not be replicated.

The telomeres at the end of chromosomes of most eukaryotic cells are replicated by a different mechanism: most telomeres are short GC-rich repeats that are added in a 5' to 3' direction by the enzyme telomerase.

Structure in sequences

Prokaryotic chromosomes have less sequence-based structure than eukaryotes. Bacteria typically have a single point (the origin of replication) from which replication starts, whereas some archaea contain multiple replication origins. The genes in prokaryotes are often organized in operons, and do not usually contain introns, unlike eukaryotes.

DNA packaging

Prokaryotes do not possess nuclei. Instead, their DNA is organized into a structure called the nucleoid. The nucleoid is a distinct structure and occupies a defined region of the bacterial cell. This structure is, however, dynamic and is maintained and remodeled by the actions of a range of histone-like proteins, which associate with the bacterial chromosome. In archaea, the DNA in chromosomes is even more organized, with the DNA packaged within structures similar to eukaryotic nucleosomes. Bacterial chromosomes tend to be tethered to the plasma membrane of the bacteria. In molecular biology application, this allows for its isolation from plasmid DNA by centrifugation of lysed bacteria and pelleting of the membranes (and the attached DNA).

Prokaryotic chromosomes and plasmids are, like eukaryotic DNA, generally supercoiled. The DNA must first be released into its relaxed state for access for transcription, regulation, and replication.

Bacterial DNA

The base composition of bacterial DNA varies between species, however, the following remarks about bacterial DNA structure are applicable to all eubacterial species. DNA in eubacterial cells is overwhelmingly in the form of a right handed B-DNA duplex. Although unusual conformation such as left handed DNA segments and cruciform structure can also exist in vivo. Non- stranded DNA can be detected in *Bacillus subtilis* during spore formation. Here, the conformation of DNA is altered from B form to the form known as A-DNA through the binding of small acid soluble proteins as the spore forms. Thus, despite this exception DNA in bacteria may be thought as being B form as illustrated in next chapter.

Bacterial DNA is negatively supercoiled

To appreciate fully the dynamic situation that obtains when genes are expressed, it is necessary to consider briefly DNA in vivo. Most of bacterial is in the form closed loops or covalently closed circular DNA (CCDNA). The DNA duplex in these loops and circles is maintained as an underwound state, and this impart torsional tension to DNA molecule. This tension may promote stand strand separation or distortion of DNA helical axis. This coiling of already coiled DNA duplex is referred to a supercoiling and when DNA supercoils in opposite of right handed clock it is said to be negative supercoiled or underwound. DNA that is over wound produce right handed supercoils and is referred as positive supercoiled. Supercoiled DNA possesses energy as consequence of their topological state. This energy is available to do thermodynamic work. The free energy of supercoiling (ΔGsc) is related in a quadratic manner to change in linking number, thus

$$\Delta Gs = (K.RT/N)\,\Delta LK$$

Where K is proportionality constant equal to 1050 for DNA molecules greater than 2kb, R is the gas constant and T the absolute temperature. This relationship tells us that relatively small changes in linking number can result in significant adjustment in the free energy supercoiling.

Bacterial Topoisomerase I

Bacterial enzymes of the DNA topoisomerase type I class that catalyze ATP-independent breakage of one of the two strands of DNA, passage of the unbroken strand through the break, and rejoining of the broken strand. These bacterial enzymes reduce the topological stress in the DNA structure by relaxing negatively, but not positively, supercoiled DNA.

Topoisomerase II

DNA topoisomerase II that catalyze ATP-dependent breakage of both strands of DNA, passage of the unbroken strands through the breaks, and rejoining of the broken strands. DNA gyrase is topoisomerase that introduce negative supercoils into DNA. Type II enzyme change the linking number of CCDNA in two steps. It derive the energy require to do this from ATP, which means that DNA supercoiling levels are indirectly modulated by cellular ATP pools. In principle, this relationship could provide a link between DNA topology and physiology of the cell. In *E.coli*, the activity of DNA gyrase is balanced by countervailing Topoisomerase I. Type I relaxes DNA by removing negative supercoils. This swivel's does not consume ATP during this reaction; energy scored in the supercoiled DNA molecule permits relaxation to proceed once topoisomerase I has made single stranded breakage in the DNA duplex.

The amount of DNA gyrase and Topoisomerase I in the cell is controlled by DNA

supercoiling at a level of transcription of their respective genes. DNA Topoisomerse I is a monomeric enzyme and the gene that code for it, topA, is activated transcriptionally by elevated level of DNA supercoiling.

DNA gyrase is made up of four subunits, two copies of A proteins encoded by gyrA, and two copies of the B protein encoded by gyrB. The promoters of gyrA and gyrB are activated by declines in supercoiling level.

Figure 2. DNA supercoiling

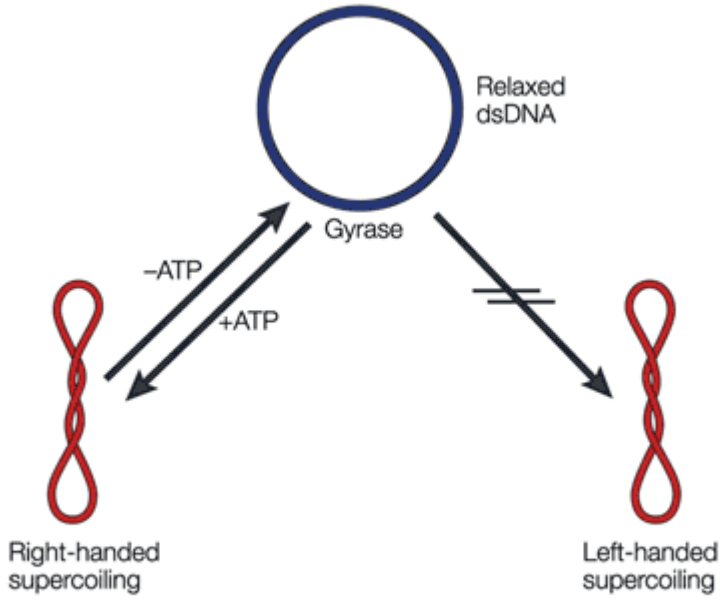
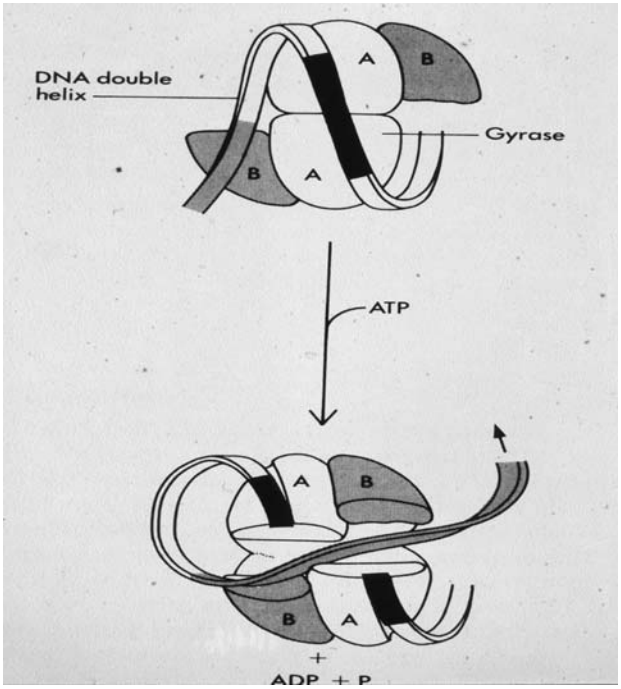


Figure 3. Mechanism action of DNA gyrase



Further evidence in support of homeostasis in supercoiling control come from genetic studies with mutant deficient in topA, the gene for topoisomerase I. Mutation in topA result in elevated levels of supercoiling in cellular DNA, presumably because gyrase has an unrestricted freedom to supercoil. topA mutant s are certainly less viable than their wild type parents. However, several independent studies have shown that topA mutants acquired additional mutations that compensate for the loss of topA gene. Many of these compensatory mutations map to the gyr genes and they restore the level of supercoiling to that of wild type.

DNA supercoiling responds to changes in growth conditions

It is now recognized that certain environmental stress experienced by bacteria result in alteration of topology of DNA and these have important consequences for the major processes of DNA. It has been discovered change in growth phase, nutrient

avability, osmolarity, and temperature produce fluctuation in linking number of DNA and therefore has great consequence in bacterial gene expression. Experiments using plasmids as reporter of DNA supercoiling have shown that when *E.coli* are grown an aerobically or exposed to osmotic stress the linking number of plasmid DNA are decreased. Changes in growth temperature produce shift in plasmid DNA supercoiling.

Nucleoid associated proteins

Much research has been carried out into the biochemical nature of the proteins associated with bacterial nucleoid and possibility that they may recognize bacterial DNA into a structure similar to eukaryotic chromatin.

HU (Histon like protein)

HU is heterodimer of 9500 KDa subunits, is basic and raped DNA without displaying overt sequence specifity. Its physical properties and aminoacid composition are reminiscent of eukaryotic histon proteins. HU has the ability to raped DNA into particles resembling nucleosomes in vitro and it has been pointed out it make 10 HU dimmers in association with 275-290 bp of DNA to form such nucleosome. It can mediate very tight DNA curvature, allowing DNA sequence as short as 99 bp to form a circle. Thus, a major biological property of HU may be to create flexibility to DNA in order to facilitate the interaction of other proteins with the DNA. Its structure is highly conserved and HU –like proteins have now been isolated from a wide range of bacteria, including *B.stearothermophilus*.

IHF (Integration host factor)

Integration host factor (IHF) is a close relative to HU and is a member the histone – like protein family. IHF is a heterodimer with physical character similar to those HU and its subunits are encoded by two unlinked genes. Genetic and invitro studies have demonstrated IHF contributes to wide variety of cellular functions including the control of transcription and site specific recombination. The manner in which IHF binds to DNA is unusual in that it use two-stranded beta ribbons to dock with the minor groove of the B-DNA helix.

H-NS

H-NS protein is another major component of the *E.coli* nucleoid. It is a neutral protein with a M.Wt. of 15,500. The cell contains about 20,000 copies of H-NS. H-NS has capacity to influence transcriptionnegatively. These effects have been shown to result from specific interactions with DNA and not simply from a general binding in the vicinity of affected promoter. This because H-NS can affect differentially transcription from two promoters located on the same plasmid. If this protein silenced transcriptionally, both promoters would have been expected to be negatively affected.

FIS

FIS is a 240 Da site specific DNA-binding protein which acts as a homodimer. It possesses a helix-turn helix motif similar to that seen in one of the major classes of DNA binding proteins. This is in contrast to the minor groove docking ribbons of HU and IHF, discussed earlier. FIS has homology to NtrC, transcription activator of sigma 54-dependent promoters. FIS was discovered originally as a factor required to

stimulate site specific inversion systems catalysed by recombinases of the invertase family and derives it name from this function. It binds to enhancer sequences in the Hin flagellar phase variation system of *Salmonella typhimurium* and to the recombinational enhancer of the Gin system of bacteriophage Mu. FIS bends DNA by about 95 degree on binding and this is probably in its biological function. DNA bending has come to be recognized as a very important feature of many regulatory system governing not just transcription but also recombination. It is a way of bringing distance sites on the same DNA molecule close together and is important in controlling the expression of many virulence factors.

Figure 4. Fluorescent in situ hybridization (FISH) of probe used against HU protein

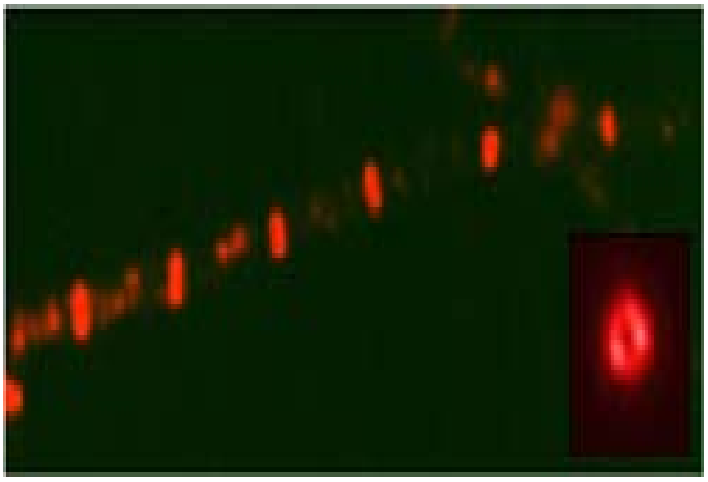
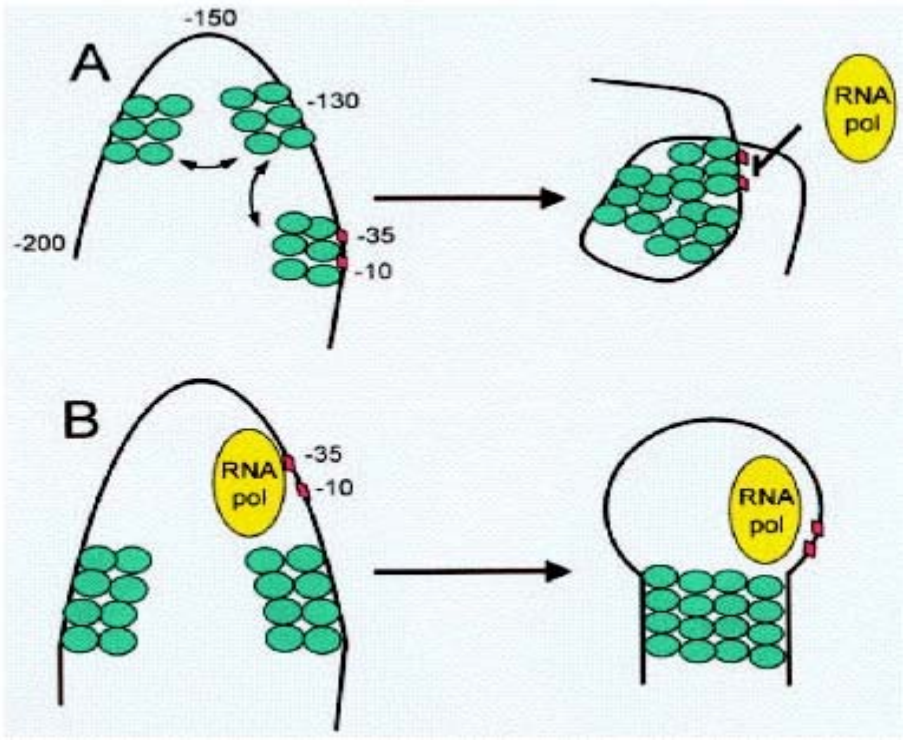


Figure 5. Mechanism action of H-NS protein



Bacterial gene expression

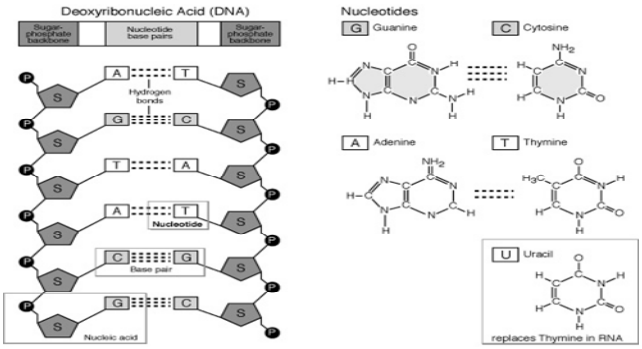
Introduction

A prokaryotic gene is expressed by transcription into mRNA and then by translation of the mRNA into protein.

In eukaryotes, a gene may contain internal regions that are not represented in Protein internal regions are removed from the RNA transcript by RNA splicing to give an mRNA that is colinear with the protein product. Each mRNA consists of a nontranslated 5' leader, a coding region, and a nontranslated 3' trailer. In comparing gene and protein, we are restricted to dealing with the sequence of DNA stretching between the points corresponding to the ends of the protein. However, a gene is not directly translated into protein, but is expressed via the production of a messenger RNA (abbreviated to mRNA), a nucleic acid intermediate actually used to synthesize a protein. Messenger RNA is synthesized by the same process of complementary base pairing used to replicate DNA, with the important difference that it corresponds to only one strand of the DNA double helix. The convention for writing DNA

sequences is that the top strand runs 5' to 3' with the sequence that is the same as

Figure 6. structure and configuration of DNA



RNA.

The process by which a gene gives rise to a protein is called gene expression. In bacteria, it consists of two stages. The first stage is transcription, when an mRNA copy of one strand of the DNA is produced. The second stage is translation of the mRNA into protein. This is the process by which the sequence of an mRNA is read in triplets to give the series of amino acids that make the corresponding protein.

A messenger RNA includes a sequence of nucleotides that corresponds with the sequence of amino acids in the protein. This part of the nucleic acid is called the coding region. But the messenger RNA includes additional sequences on either end; these sequences do not directly represent protein. The 5' nontranslated region is called the leader, and the 3' nontranslated region is called the trailer.

The gene includes the entire sequence represented in messenger RNA. Sometimes mutations impeding gene function are found in the additional, noncoding regions, confirming the view that these comprise a legitimate part of the genetic unit. It includes the sequence coding for that protein, but also includes sequences on either side of the coding region. Several processes are required to express the protein product of a gene.

In eukaryotes transcription occurs in the nucleus, but the RNA product must be transported to the cytoplasm in order to be translated. For the simplest eukaryotic genes (just like in bacteria) the transcript RNA is in fact the mRNA. But for more complex genes, the immediate transcript of the gene is a pre-mRNA that requires processing to generate the mature mRNA. This results in a spatial separation between transcription (in the nucleus) and translation (in the cytoplasm). (Several processes are required to express the protein product of a gene.

The most important stage in processing is RNA splicing. Many genes in eukaryotes and majority in higher eukaryotes contain internal regions that do not code for protein. The process of splicing removes these regions from the pre-mRNA to generate an RNA that has a continuous open reading frame. Other processing events that occur at this stage involve the modification of the 5' and 3' ends of the pre-mRNA. Translation is accomplished by a complex apparatus that includes both protein and RNA components. The actual "machine" that undertakes the process is the ribosome, a large complex that includes some large RNAs (ribosomal RNAs, abbreviated to rRNAs) and many small proteins. The process of recognizing which amino acid corresponds to a particular nucleotide triplet requires an intermediate transfer RNA)abbreviated to tRNA; there is at least one tRNA species for every amino acid. Many ancillary proteins are involved. We describe translation in Molecular Biology 2.5 Messenger RNA, but note for now that the ribosomes are the large structures in Figure 1.38 that move along the mRNA.

The important point to note at this stage is that the process of gene expression involves RNA not only as the essential substrate, but also in providing components of the apparatus. The rRNA and tRNA components are coded by genes and are

generated by the process of transcription (just like mRNA, except that there is no subsequent stage of translation.

Genes are DNA

Proteins are trans-acting but sites on DNA are cis-acting. Cis configuration describes two sites on the same molecule of DNA trans configuration of two sites refers to their presence on two different molecules of DNA (chromosomes A cis-acting site affects the activity only of sequences on its own molecule of DNAor RNA; this property usually implies that the site does not code for protein.

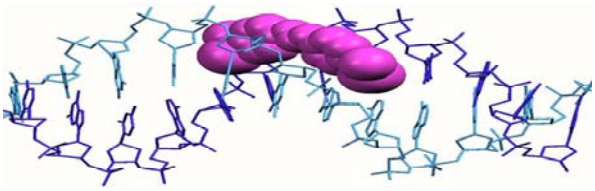
All gene products (RNA or proteins) are trans-acting. They can act on any copy of a gene in the cell. cis-acting mutations identify sequences of DNA that are targets for recognition by trans-acting products. They are not expressed as RNA or protein and affect only the contiguous stretch of DNA.

A crucial step in the definition of the gene was the realization that all its parts must be present on one contiguous stretch of DNA. In genetic terminology, sites that are located on the same DNA are said to be in cis. Sites that are located on two different molecules of DNA are described as being in trans. So two mutations may be in cis on the same DNA or in trans (on different DNAs). The complementation test uses this concept to determine whether two mutations are in the same gene since mutation in cisgenes cannot complement each other.

We may now extend the concept of the difference between cis and trans effects from defining the coding region of a gene to describing the interaction between regulatory elements and a gene Suppose that the ability of a gene to be expressed is controlled

by a protein that binds to the DNA close to the coding region.

Figure 7, Role of DNA binding protein in DNA replication

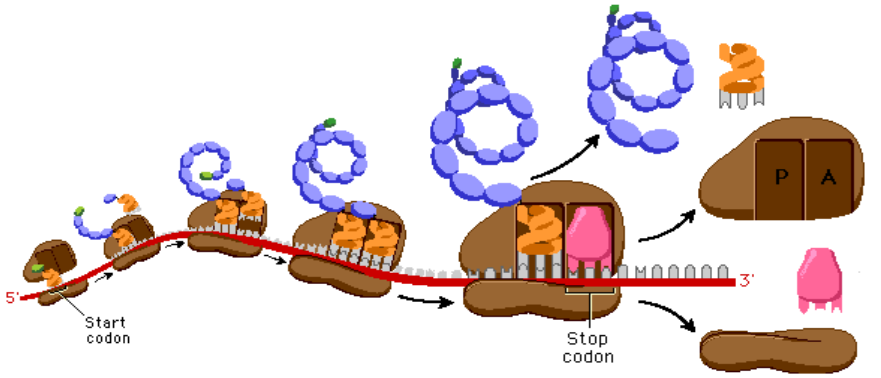


Messenger RNA can be synthesized only when the protein is bound to the DNA. Now suppose that a mutation occurs in the DNA sequence to which this protein binds, so that the protein can no longer recognize the DNA. As a result, the DNA can no longer be expressed. So, a gene can be inactivated either by a mutation in a control site or by a mutation in a coding region. The mutations cannot be distinguished genetically, because both have the property of acting only on the DNA sequence of the single allele in which they occur. They have identical properties in the complementation test, and a mutation in a control region is therefore defined as comprising part of the gene in the same way as a mutation in the coding region.

A mutation of this sort is said to be trans-acting. Reversing the argument, if a mutation is trans-acting, we know that its effects must be exerted through some diffusible product (typically a protein) that acts on multiple targets within a cell. But if a mutation is cis-acting, it must function via affecting directly the properties of the contiguous DNA, which means that it is not expressed in the form of RNA or protein.

Translation consists of three stages initiation, elongation and termination.

Figure 8. Stages of protein synthesis.



Genetic information can be provided by DNA or RNA

The central dogma describes the basic nature of genetic information: sequences of nucleic acid can be perpetuated and interconverted by replication, transcription, and reverse transcription, but translation from nucleic acid to protein is unidirectional, because nucleic acid sequences cannot be retrieved from protein sequences A retrovirus is an RNA virus with the ability to convert its sequence into DNA by reverse transcription. Reverse transcription is synthesis of DNA on a template of RNA. It is accomplished by the enzyme reverse transcriptase. Cellular genes are DNA, but viruses and viroids may have genes of RNA.

DNA is converted into RNA by transcription, and RNA may be converted into DNA by reverse transcription. The translation of RNA into protein is unidirectional.

The central dogma defines the paradigm of molecular biology. Genes are perpetuated as sequences of nucleic acid, but function by being expressed in the form of proteins. Replication is responsible for the inheritance of genetic information. Transcription and translation are responsible for its conversion from one form to another.

The perpetuation of nucleic acid may involve either DNA or RNA as the genetic material. Cells use only DNA. Some viruses use RNA, and replication of viral RNA occurs in the infected cell.

Figure 9.X-ray crystallography of DNA polymerase enzyme.

The expression of cellular genetic information usually is unidirectional. Transcription of DNA generates RNA molecules that can be used further only to generate protein sequences; generally they cannot be retrieved for use as genetic information. Translation of RNA into protein is always irreversible.

These mechanisms are equally effective for the cellular genetic information of prokaryotes or eukaryotes, and for the information carried by viruses. The genomes of all living organisms consist of duplex DNA. Viruses have genomes that consist of DNA or RNA; and there are examples of each type that are double-stranded (ds) or single-stranded (ss). Details of the mechanism used to replicate the nucleic acid vary among the viral systems, but the principle of replication via synthesis of complementary strands remains the same.

Cellular genomes reproduce DNA by the mechanism of semi-conservative replication. Double-stranded virus genomes, whether DNA or RNA, also replicate by using the individual strands of the duplex as templates to synthesize partner strands. Viruses with single-stranded genomes use the single strand as template to synthesize a complementary strand; and this complementary strand in turn is used to synthesize its complement, which is, of course, identical with the original starting strand.

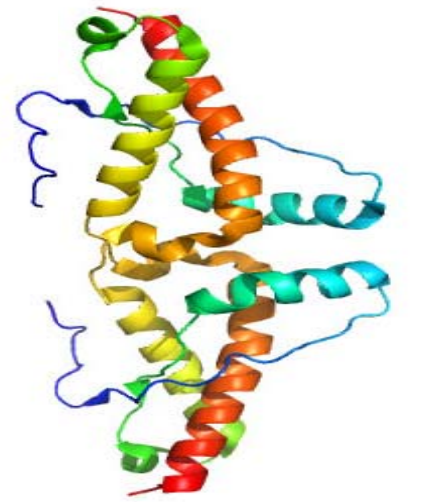
Replication may involve the formation of stable double-stranded intermediates or use double-stranded nucleic acid only as a transient stage. The restriction to unidirectional transfer from DNA to RNA is not absolute. It is overcome by the retroviruses, whose genomes consist of single-stranded RNA molecules. During the infective cycle, the RNA is converted by the process of reverse transcription into a single-stranded DNA, which in turn is converted into a double-stranded DNA. This duplex DNA becomes part of the genome of the cell, and is inherited like any other gene. So reverse transcription allows a sequence of RNA to be retrieved and used as genetic information.

The existence of RNA replication and reverse transcription establishes the general principle that information in the form of either type of nucleic acid sequence can be converted into the other type. In the usual course of events, however, the cell relies on the processes of DNA replication, transcription, and translation. But on rare occasions (possibly mediated by an RNA virus), information from a cellular RNA is converted into DNA and inserted into the genome. Although reverse transcription plays no role in the regular operations of the cell, it becomes a mechanism of potential importance when we consider the evolution of the genome.

The same principles are followed to perpetuate genetic information from the massive genomes of plants or amphibians to the tiny genomes of mycoplasma and the yet smaller genetic information of DNA or RNA viruses. Figure 1.45 summarizes some examples that illustrate the range of genome types and sizes throughout the range of organisms, with genomes varying in total content over a 100/0000 fold range, a common principle prevails. The DNA codes for all the proteins that the cell(s) of the organism must synthesize; and the proteins in turn (directly or indirectly) provide the functions needed for survival. A similar principle describes the function of the genetic information of viruses, whether DNA or RNA. The nucleic acid codes for the protein(s) needed to package the genome and also for any functions additional to those provided by the host cell that are needed to reproduce the virus during its infective cycle. (The smallest virus, the satellite tobacco necrosis virus [STNV], cannot replicate independently, but requires the simultaneous presence of a "helper" virus [tobacco necrosis virus, TNV], which is itself a normally infectious virus.

Some hereditary agents are extremely small. A viroid is a small infectious nucleic acid that does not have a protein coat. Virion is the physical virus particle (irrespective of its ability to infect cells and reproduce. A subviral pathogen is an infectious agent that is smaller than a virus, such as a viroid. Scrapie is an infective agent made of protein. A prion is a proteinaceous infectious agent, which behaves as an inheritable trait although it contains no nucleic acid. Examples are PrPSc, the agent of scrapie in sheep and bovine spongiform encephalopathy, and Psi, which confers an inherited state in yeast. PrP is the protein that is the active component of the prion that causes scrapie and related diseases. The form involved in the disease is called PrPSc .Some very small hereditary agents do not code for protein but consist of RNA or of protein that has hereditary properties.

Figure 10.X-ray crtstallography of PrP protein Sheep with scarpie



Viroids are infectious agents that cause diseases in higher plants. They are very small circular molecules of RNA. Unlike viruses, where the infectious agent consists of a virion, a genome encapsulated in a protein coat, the viroid RNA is itself the infectious agent. The viroid consists solely of the RNA, which is extensively but imperfectly base paired, forming a characteristic rod like. Mutations that interfere with the structure of the rod reduce infectivity in infected cells. Its sequence is faithfully perpetuated in its descendants. Viroids fall into several groups. A given viroid is identified with a group by its similarity of sequence with other members of the group. For example, four viroids related to PSTV (potato spindle tuber viroid) have 70-83% similarity of sequence with it.

Different isolates of a particular viroid strain vary from one another, and the change may affect the phenotype of infected cells. For example, the mild and severe strains of PSTV differ by three nucleotide substitutions. Viroids resemble viruses in having heritable nucleic acid genomes. They fulfill the criteria for genetic information. Yet viroids differ from viruses in both structure and function. They are sometimes called

subviral pathogens. Viroid RNA does not appear to be translated into protein. So it cannot itself code for the functions needed for its survival. This situation poses two questions. How does viroid RNA replicate? and how does it affect the phenotype of the infected plant cell.?

Replication must be carried out by enzymes of the host cell, subverted from their normal function. The heritability of the viroid sequence indicates that viroid RNA provides the template.

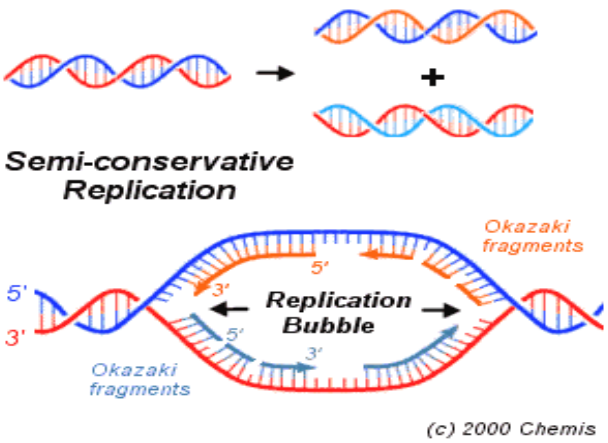
Viroids are presumably pathogenic because they interfere with normal cellular processes. They might do this in a relatively random way, for example, by sequestering an essential enzyme for their own replication or by interfering with the production of necessary cellular RNAs. Alternatively, they might behave as abnormal regulatory molecules, with particular effects upon the expression of individual genes.

An even more unusual agent is scrapie, the cause of a degenerative neurological disease of sheep and goats. The disease is related to the human diseases of kuru and Creutzfeldt-Jakob syndrome, which affect brain function. The infectious agent of scrapie does not contain nucleic acid. This extraordinary agent is called a prion (proteinaceous infectious agent). It is hydrophobic glycoprotein, PrP. PrP is coded by a cellular gene (conserved among the mammals) that is expressed in normal brain. The protein exists in two forms. The product found in normal brain is called PrPc. It is entirely degraded by proteases. The protein found in infected brains is called PrPsc. It is extremely resistant to degradation by proteases. PrPc is converted to PrPsc by a modification or conformational change that confers protease-resistance, and which has yet to be fully defined.

As the infectious agent of scrapie, PrPsc must in some way modify the synthesis of its normal cellular counterpart so that it becomes infectious instead of harmless

Prions cause diseases in mammals. Mice that lack a PrP gene cannot be infected to develop scrapie, which demonstrates that PrP is essential for development of the disease.

At first the origin of replication must be recognize by specific proteins in origin two DNA strand get separated.and



Properties of Oric.in bacteria

1- It should contain specific sequences recognize by initiation proteins
2- It should be negative supercoil
3- It should be rich in AT sequence

Molecular techniques in bacteriology

What is PCR?

Sometimes called "molecular photocopying," the polymerase chain reaction (PCR) is a fast and inexpensive technique used to "amplify" - copy - small segments of DNA. Because significant amounts of a sample of DNA are necessary for molecular and genetic analyses, studies of isolated pieces of DNA are nearly impossible without PCR amplification. Often heralded as one of the most important scientific advances in molecular biology, PCR revolutionized the study of DNA to such an extent that its creator, Kary B. Mullis, was awarded the Nobel Prize for Chemistry in 1993.from the National Human Genome Research Institute.

What is it used for?

Once amplified, the DNA produced by PCR can be used in many different laboratory procedures has -

Most mapping techniques in the Human Genome Project rely on PCR.

PCR is integral in a number of new laboratory and clinical techniques, including DNA fingerprinting (think CSI and catching criminals).

Diagnosing disease and genetic disorders.

Detection of bacteria and viruses in the environment.

Analysis of microbial communities.

How does it work?

To amplify a segment of DNA using PCR, the sample is first heated so the DNA denatures, separates into two pieces of single-stranded DNA. Next, an enzyme called "Taq polymerase" synthesizes - builds - two new strands of DNA, using the original strands as templates. This process results in the duplication of the original DNA, with each of the new molecules containing one old and one new strand of DNA. Then each of these strands can be used to create two new copies, and so on, and so on. The cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times, leading to more than one billion exact copies of the original DNA

segment. The entire cycling process of PCR is automated and can be completed in just a few hours. It is directed by a machine called a thermocycler, which is programmed to alter the temperature of the reaction every few minutes to allow DNA denaturing and synthesis.

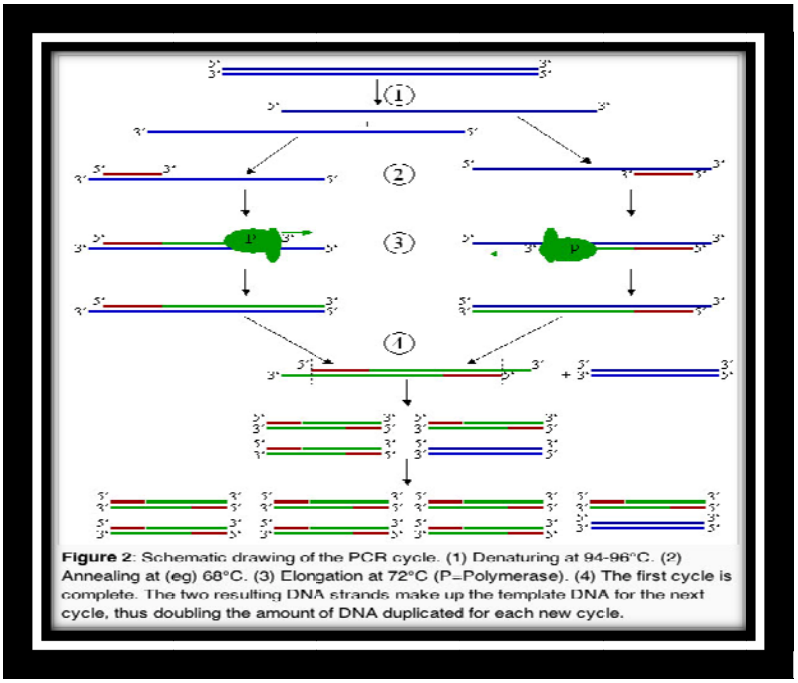


Figure 2: Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at (eg) 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle.

Figure 11. Instrumentation used in PCR -

A thermocycler or PCR machine is a laboratory apparatus used for PCR. The device has a thermal block with holes where tubes with the PCR reaction mixtures can be

inserted. The cycler then rises and lowers the temperature of the block in discrete, pre-programmed steps. Thermal cyclers are equipped with hot bonnet, which is a heated plate that presses against the lids of the reaction tubes. This prevents condensation of water from the reaction mixtures to the insides of the lids and makes it unnecessary to use PCR oil.

Reverse transcription- polymerase chain reaction (RT-PCR)

The starting template for a PCR reaction can be DNA or RNA. DNA is usually the appropriate template for studying the genome of the cell or tissue (as in inherited genetic diseases, somatic mutation in a tumor, or somatic rearrangement in lymphocytes) and for the detection of DNA viruses.

For information on gene expression in a cell or tissue, or the presence of genomic RNA in a retrovirus such as HIV, RNA is the appropriate template. RNA can be better than genomic DNA for detecting structural changes in long genes, since amplifying the spliced RNA transcript instead of the genomic sequence greatly reduces the length of DNA to be handled without losing any of the coding regions where clinically significant deletions may be expected.

RT-PCR combines cDNA synthesis from RNA templates with PCR to provide a rapid, sensitive method for analyzing gene expression (Figure 3). RT-PCR is used to detect or quantify the expression of mRNA, often from a small concentration of target RNA.

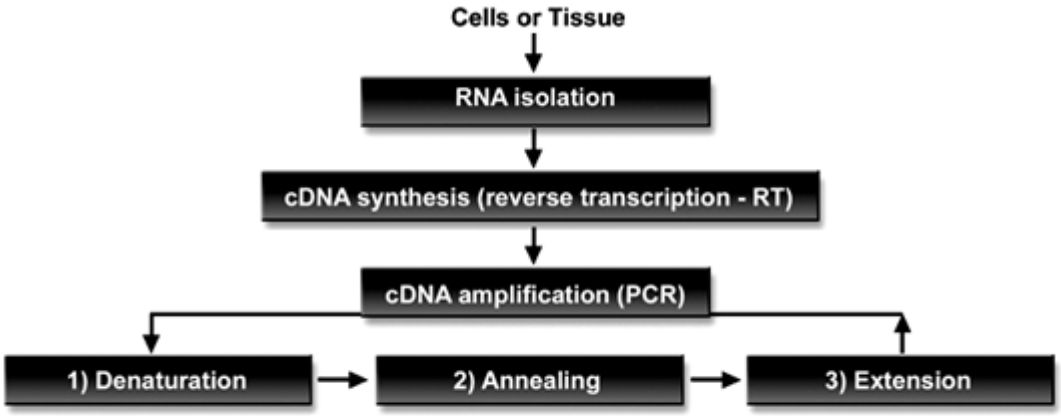


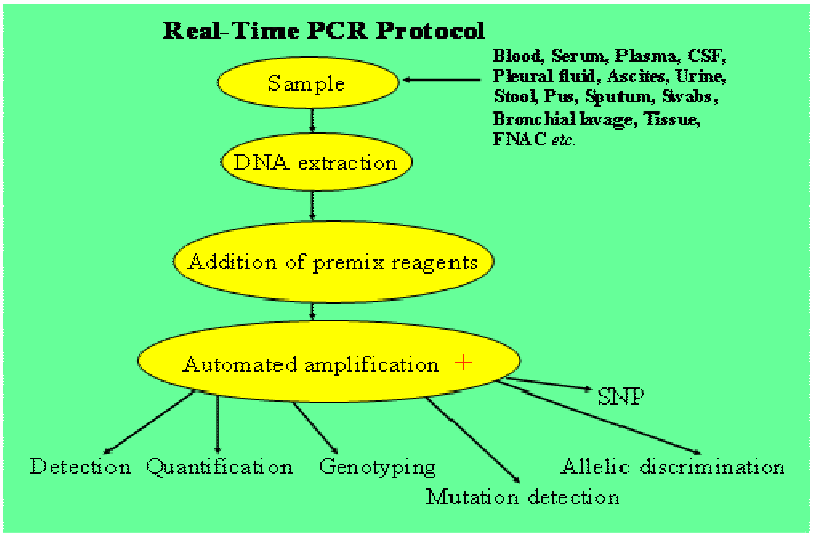
FIGURE 3 - Schematic diagram of RT-PCR showing that RNA isolated from cells or tissue is used as substrate in reverse transcription for synthesis of cDNA that will serve as template for amplification by PCR.

The template for RT-PCR can be total RNA or poly $(A)^+$ selected RNA. RT reactions can be primed with random primers, oligo(dT), or a gene-specific primer (GSP) using a reverse transcriptase. RT-PCR can be carried out either in two-step or one-step formats. In two-step RT-PCR, each step is performed under optimal conditions. cDNA synthesis is performed first in RT buffer and one tenth of the reaction is removed for PCR[50,51]. In one-step RT-PCR, reverse transcription and PCR take place sequentially in a single tube under conditions optimized for both RT and PCR.

Real time PCR

Definition

First significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product. Usually the protocol followed is depicted in Figure 12 as shown below.



Real time PCR or quantitative PCR is a variation of the standard PCR technique used to quantify DNA or messenger RNA (mRNA) in a sample. Using sequence specific primers, the relative number of copies of a particular DNA or RNA sequence can be determined. We use the term relative since this technique tends to be used to compare relative copy numbers between tissues, organisms, or different genes relative to a specific housekeeping gene. The quantification arises by measuring the amount of amplified product at each stage during the PCR cycle. DNA/RNA from genes with higher copy numbers will appear after fewer melting, annealment, extension PCR cycles. Quantification of amplified product is obtained using fluorescent probes and specialized machines that measure fluorescence while performing temperature changes needed for the PCR cycles.

Various Probe formats

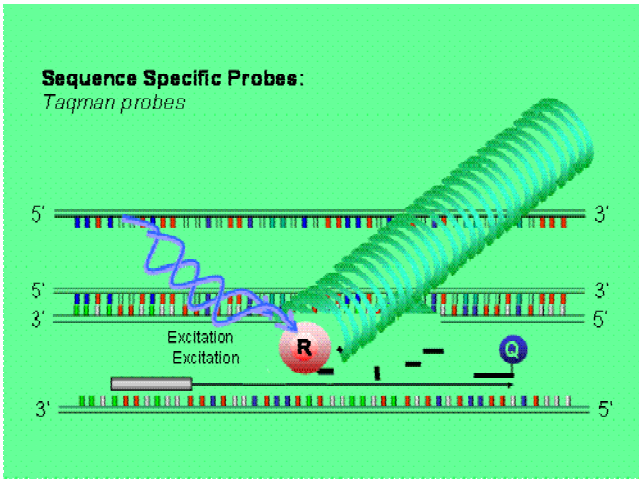
There are three main fluorescence-monitoring systems for DNA amplification

(1) Hydrolysis probes

Hydrolysis probes include TaqMan probes, molecular beacons. They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples.*TaqMan probes* are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10 ^{o}C higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base (TaqMan MGB probes have a non-fluorescent quencher and minor groove binder at the 3' end). When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing. Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye 22. This ends the activity of quencher and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of

the product. One specific requirement for fluorogenic probes is that there be no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimization.

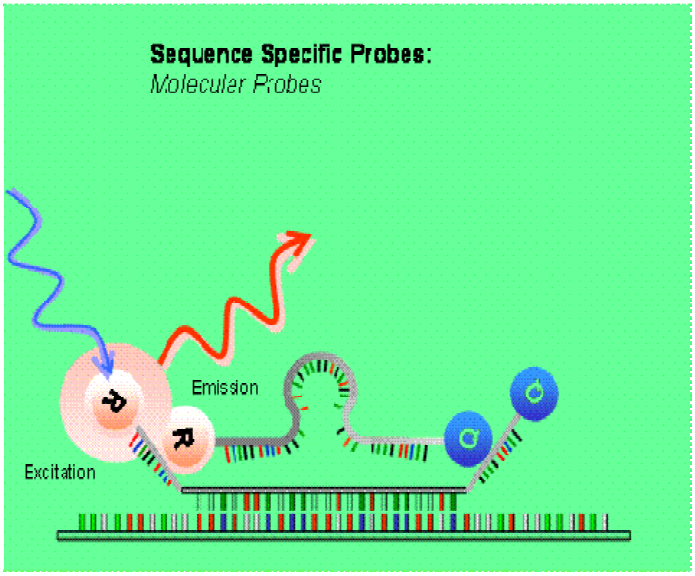
Figure 13. Mechanism of real time PCR



Molecular beacons also contain fluorescent (FAM, TAMRA, TET, ROX) and quenching dyes (typically DABCYL) at either end but they are designed to adopt a hairpin structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur. They have two arms with complementary sequences that form a very stable hybrid or stem. The close proximity of the reporter and the quencher in this hairpin configuration suppresses reporter fluorescence. When the beacon hybridizes to the target during the annealing step, the reporter dye is separated from the quencher and the reporter fluoresces. Molecular beacons remain intact during PCR and must rebind to target every cycle for fluorescence emission. This will correlate to the amount of PCR product available. All real-time PCR chemistries allow detection of multiple DNA species (multiplexing) by designing each probe/beacon with a spectrally unique fluor/quench pair, or if SYBR green is

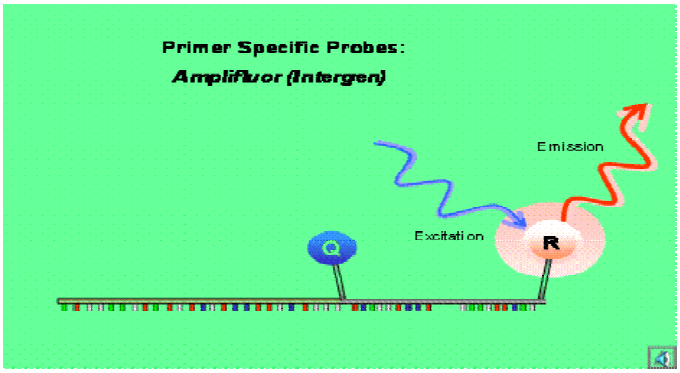
used by melting curve analysis. By multiplexing, the target(s) and endogenous control can be amplified in single tube for qPCR purposes.

Figure 14. Sequence specific probe



With Scorpion primer/probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is

Figure 15. Primer specific probe



Observed.

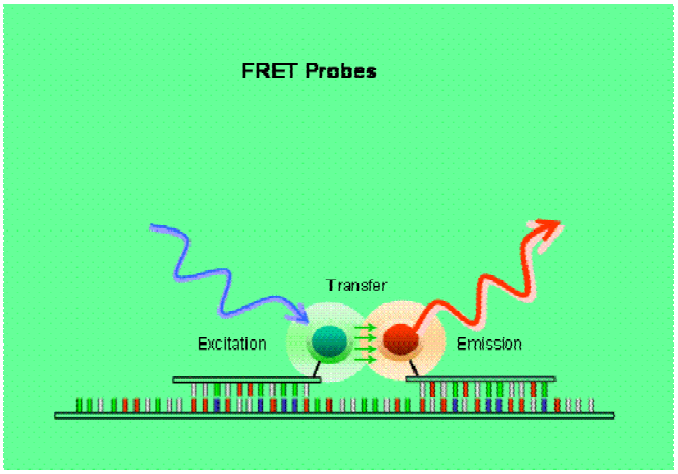
(2) Hybridizing probes

Fret probes

FRET Probes rely on the transfer of Energy from one fluorescent dye to another. Two separate sequence specific oligos are fluorescently labeled. The upstream probe has a donor molecule on the 3'- end and the downstream probes has an acceptor molecule on the 5'-end. The probes are designed so that they hybridize adjacently to each other on the target sequence and bring the donor and acceptor fluorophores in close proximity. Once the probes are hybridized, the donor and acceptor fluorescent molecules are in close proximity to one another. This allows for transfer of energy from the donor to the acceptor fluorophore, which emits a signal of a different wavelength. Either the decrease in the fluorescence of the donor or the increase in fluorescence of the acceptor can be detected. Therefore, only when both probes are bound is fluorescence detectable. FRET probes do allow for melt curve

analysis. They are extremely useful for Genotyping, SNP detection and other mutation detections.

Figure 16. FRET probe mechanism

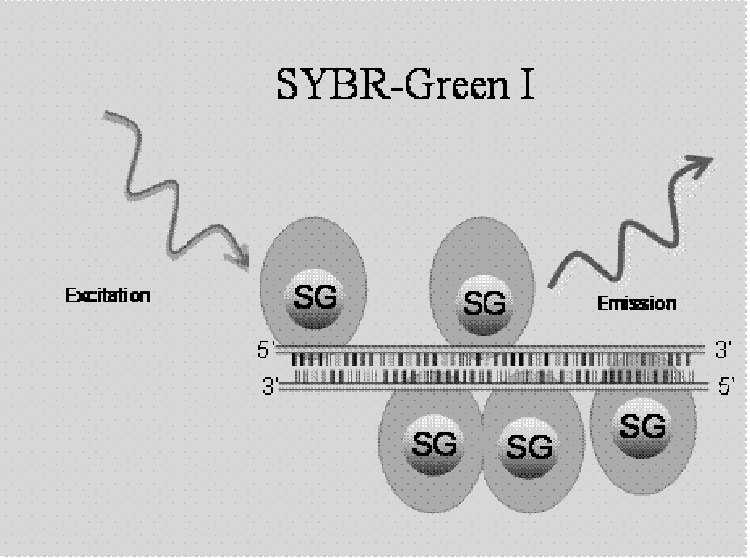


(3) DNA-binding agents

The cheaper alternative is the double-stranded DNA binding dye chemistry, which quantitates the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (SYBR-green I or ethidium bromide). It does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a b fluorescent signal upon binding to double-stranded DNA Disadvantages of SYBR green-based real-time PCR include the requirement for extensive optimization. Furthermore, non-specific amplifications require follow-up assays (melting point or dissociation curve analysis) for amplicon identification. The method has been used in HFE-C282Y genotyping. Another controllable problem is that longer amplicons create a ber signal (if combined with other factors, this may cause CDC camera saturation.

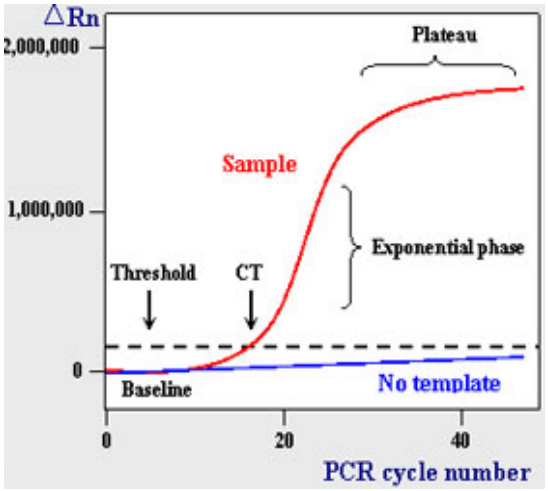
Normally SYBR green is used in single-plex reactions, however when coupled with melting curve analysis, it can be used for multiplex reactions.

Figure 17. Example of syber green mechanism.



Quantification

The diagram above shows a typical reading from a single PCR cycle in a real time PCR machine. The vertical axis represents copy number (arbitrary units) and the horizontal axis shows the PCR cycle number (ie, how many melting/annealment/extension cycles have occurred). The dotted threshold line is an arbitrary value, usually about 0.1 and is the "copy number" used to determine Ct. The lower a Ct value, the more copies are present in the specific sample. When plotted on a linear scale, as above, the curve has a sigmoidal course with an exponential phase and a plateau phase. The plateau phase is really determined by the amount of primer in the master mix rather than the nucleotide template.



Usually the vertical scale is plotted in a logarithmic fashion, allowing the intersection of the plot with the threshold to be linear and more easily visualized. Theoretically, the amount of DNA doubles every cycle during the exponential phase, but this can be affected by the efficiency of the primers used. A negative control is always performed with no template to show a lack of intrinsic fluorescence. A positive control using a housekeeping gene that is relatively abundant in all cell types is also performed to allow for comparisons between samples. Typical housekeeping genes include 18S rRNA, GAPDH, and actin. When real time PCR is combined with reverse transcriptase PCR (RT-PCR), mRNA can be quantified for an assessment of relative gene expressions between tissues or genes. The amount of DNA/RNA is determined by comparing the results to a standard curve produced by serial dilutions of a known amount of DNA/RNA. Some sort of reporter method is required to be able to quantify amplified product after each PCR cycle. A popular method is the use of double-stranded DNA dyes. These dyes no selectively bind to all double-stranded DNA, resulting in fluorescence. dsDNA dyes such as SYBR Green are nonselective such that they will bind to any dsDNA, including primer dimmers. Another method of PCR product quantification is the use of a fluorescent reporter probe.

Electrophoresis

Introduction

There two main techniques by which electrophoresis is achieved today: gel and

capillary electrophoresis, both of which are based on the same principle of size and charge based fragment separation. This separation is facilitated by the negative charge present on the DNA fragments due to the release of positive hydrogen ions from the phosphate groups that constitute the 'backbone' of the molecule in the presence of ionic buffer solutions such as Tris Borate Ethylenediamine tetra–acetic acid (EDTA) (TBE) or Tris Acetate EDTA (TAE).

Figure 18. Gel electrophoresis

Gel electrophoresis can be performed in a horizontal or vertical plane, using agarose or polyacrylamide gel as a separation medium. Despite the variation used, the presence of an ionic buffer solution and constant electrical charge across the gel is a ubiquitous necessity in achieving separation of the DNA.

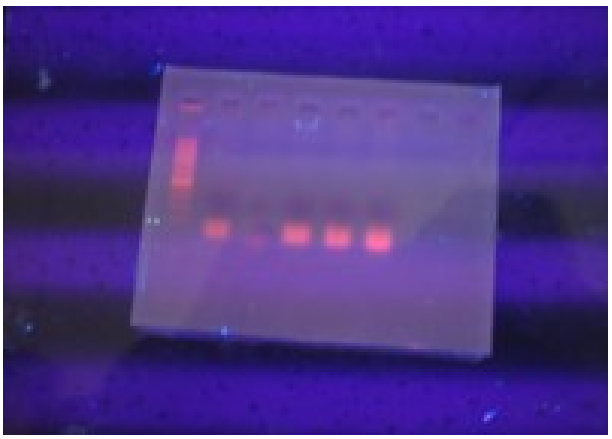
Gels are usually made in the laboratory by pouring the liquid form of either agarose or polyacrylamide into a pre-formed solid mould; a comb is inserted at one end to create wells, into which the DNA of interest will be loaded for separation. The gel is then allowed to solidify before the comb is removed and the gel is transferred into a buffer-containing electrophoresis tank where the DNA can be loaded into the gel wells and separation can take place.

Agarose gels

The choice of gel used is largely dependent upon the size and spacing of the DNA fragments under analysis. Agarose is a polysaccharide which, together with

agaropectin, forms the seaweed-derived, gelatinous substance agar. When set, the polysaccharide strands form a matrix structure through which DNA molecules can travel when a charge is present at either end of the gel.

Figure 19. Appearance of DNA after staining on gel.



The pore sizes within this matrix are considered relatively large at approximately 100-300nm depending upon the concentration of agarose, and as such it does not allow for accurate resolution of closely sized DNA fragments. Agarose gels are generally used when larger fragments, in the region of 500-20,000bp, are required to be visualized.

They can also be used to assess the quality of extracted DNA, with degraded template producing a smear when run on an agarose gel as opposed to a tight band of high molecular weight for high quality samples. Agarose gel electrophoresis is used for fragment separation during the DNA fingerprinting method described above.

Polyacrylamide gels

Polyacrylamide gels are made by inducing polymerisation of acrylamide and bisacrylamide monomers in a process initialised by the presence of Ammonium Persulphate and TEMED (N, N, N', N'-tetramethylethylenediamine).

The use of an artificial gel matrix in place of the naturally extracted agarose produces smaller pore sizes in the gel matrix at approximately 10-20nm in a typical gel. This pore size reduction, along with optimised running conditions, can allow for highly accurate resolution of similarly sized DNA fragments and under denaturing

conditions achieves resolution of single base-pair size differences.

Figure 20. Detection of sequence on gel.

This level of accuracy led to polyacrylamide gels being employed for separation of amplified STR markers during DNA profiling development but has now been largely replaced by more sensitive capillary electrophoresis technologies.

Polyacrylamide gel electrophoresis (PAGE) is conducted in a manner very similar to agarose gel analysis. The un-polymerised solution is poured between two closely spaced glass plates, a gel comb is inserted to create the wells into which the DNA will be loaded and the solution is allowed to polymerise or set over 1-2 hours.

Once set, the gel is moved to the running apparatus where a buffer is placed at the top and bottom of the gel, the DNA of interest is loaded into the wells created during polymerization, and separation occurs in the same way as for agarose gels when a fixed current is applied across the gel apparatus.

Capillary electrophoresis

Capillary electrophoresis is very amenable to automated and high-throughput processing. Capillary wells created during casting of the gel. For CE, the DNA sample is loaded into the separation medium by electrokinetic injection whereby a positive charge is applied to draw the negatively charged DNA into the capillary. This method of loading requires fewer operators.

Another major difference is in the method of loading the DNA sample of interest in to the capillary. With gel systems the samples mut be carefully loaded by an operator into the Capillaries are made of fused silica and have an internal diameter of only 50-100μm and can be 25-100cm in length. Similarly to gelelectrophoresis, size separation is achieved via use of buffer solutions and application of positive and negative charges at either end of the

Capillary electrophoresis (CE) is more accurately described as a variation on the more established gel electrophoresis methods ather than a new technique in its own right. The main difference in the two electrophoretic techniques is the use of a capillary containing a polymer solution such as hydroxyethylcellulose in place of the traditional physical gel.

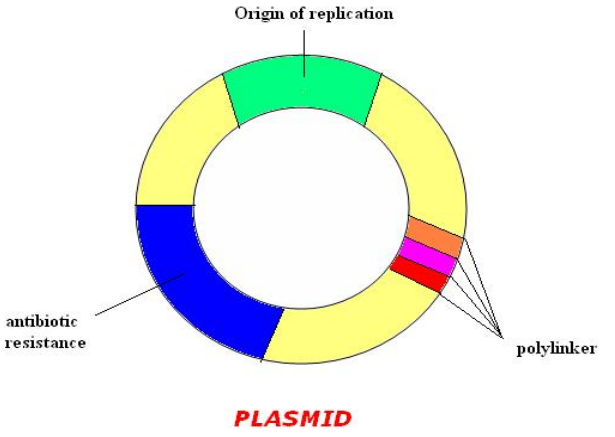
Bacterial Cloning

Introduction

A plasmid is an accessory chromosomal DNA that is naturally present in bacteria.

Some bacteria cells can have no plasmids or several copies of one. They can replicate independently of the host chromosome. Plasmids are circular and double stranded. They carry few genes and their size ranges from 1 to over 200 kilobase pairs. Some functions of their genes include: providing resistance to antibiotics, producing toxins and the breakdown of natural products. However, plasmids are not limited to bacteria; they are also present in some eukaryotes (e.g., circular, nuclear plasmids in Dictyostelium purpureum). A plasmid is a circular, double stranded DNA that is usually found in bacteria (however it does occur in both eukarya and prokarya). It replicates on its own (without the help of chromosomal DNA)and are used frequently in recombinant DNA research in order to replicate genes of interest. Some plasmids can be implanted into a bacterial or animal chromosome in which it becomes a part of the cell's genome and then reveals the gene of interest as a phenotype. This is how much research is done today for gene identification. Plasmids contain three components: an origin of replication, a polylinker to clone the gene of interest (called multiple cloning site where the restriction enzymes cleave), and an antibiotic resistance gene (selectable marker).

Figure 21. Structure of a plasmid.

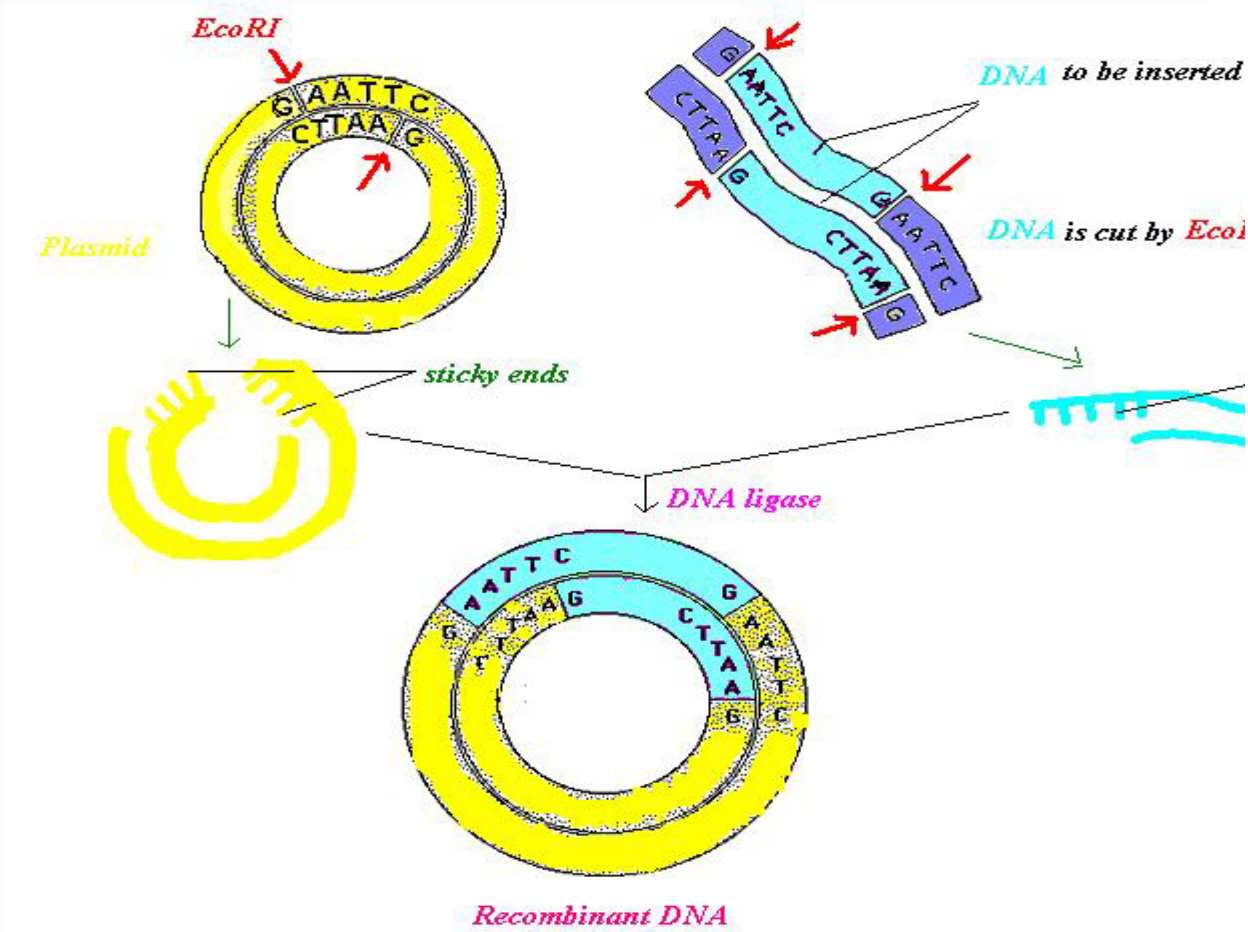


Plasmids are usually isolated before they are used in recombinant techniques. Alkaline lysis is the method of choice for isolating circular plasmid DNA. This process is quick and reliable. You first obtain the cell that has the plasmid of interest and lyse it with alkali. This step is then followed by extracting the plasmid. The cell fragments are precipitated by using SDS and potassium acetate. This is spun down, and the pellet (cell/cell fragments) is removed. Next, the plasmid DNA is precipitated from the supernatant with the use of isopropanol. The plasmid is then suspended in buffer. Akaline lysis can give you different amounts of plasmid depending if it's a mini-, midi-, or maxi- prep.

Plasmids can be related to viruses because they can be independent life-forms due to their ability to self-replicate inside their host. Though they may be viewed as independent life-forms, they have a sense of dependency on their host. A plasmid and its host tend to have a symbiotic relationship. Plasmids can give their hosts needed packages of DNA carrying genes that can lead to mutual survival during tough times. Providing its host with such genetic information, plasmid allows the host

to survive and at the same time allows itself to continue living in the host for generations.

Figure 22. Mechanism of cloning.



Plasmids are used as vectors to clone DNA in bacteria. One example of a plasmid used for DNA cloning is called pBR322 Plasmid. The pBR322 plasmid contains a gene that allows the bacteria to be resistant to the antibiotics tetracycline and amipicillin. To use pBR322 plasmid to clone a gene, a restriction endonuclease first cleaves the plasmid at a restriction site. pBR322 plasmid contains three restriction sites: PstI, SalI and ecoRI. The first two restriction sites are located within the gene

that codes for ampicillin and tetracycline resistance, respectively. Cleaving at either restriction site will inactivate their respective genes and antibiotic resistance. The target DNA is cleaved with a restriction endonuclease at the same restriction site. The target DNA is then annealed to the plasmid using DNA ligase. After the target DNA is incorporated into the plasmid, the host cell is grown in a environment containing ampicillin or tetracycline, depending on which gene was left active. Many copies of the target DNA is created once the host is able to replicate.

Another plasmid used as a vector to clone DNA is called pUC18 plasmid. This plasmid contains a gene that makes the host cell ampicillin resistant. It also contains a gene that allows it produce beta-galactosidase, which is an enzyme degrades certain sugars. The enzyme produces a blue pigment when exposed to a specific substrate analog. This allows the host to be readily identified. The gene for beta-galactosidase contains a polylinker region that contains several restriction sites. The pUC18 plasmid can be cleaved by several different restriction endonucleases which provide more versatility. When the polylinker sequence is cleaved and the target DNA is introduced and ligated, this inactivates the gene that codes for beta-galactosidase and the enzyme will not be produced. The host cell will not produce a blue pigment when exposed to the substrate analog. This allows the recombinant cells to be readily identified and isolated.

Cloning is a method of recombining genes in order to take advantage of a bacteria's native ability to recreate plasmids. Engineered plasmids can be used to clone genetic material of up to 10,000 base pairs, the amount of genetic material is limited by the size of the plasmid. Because of the repetition of expressive genes within bacterial plasmids, it is possible to remove repeated genetic materials of the plasmid

and replace it with desired traits. Most pre-engineered plasmids procured for laboratory use already contain an antibiotic resistance gene, polylinker site, and an origin of replication. The polylinker site is engineered to allow multiple unique cleaving sites that will allow needed DNA fragmentation. The origin of replication will mimic the genetic material of the bacteria that will be used for cloning. Once the plasmid is acquired, the polylinker will be cleaved at two sites using specific endonucleases. Afterwards, the wanted DNA will also be cleaved from a different source with a different endonuclease. The cleaved DNA is sometimes amplified with a polymerase chain reaction. The desired DNA trait will be inserted into the now empty polylinker site. This replacement of the polylinker site with desired genetic traits is termed a cassette mutagenesis. The newly created plasmid will be mixed with bacteria, which will then be heat shocked or electric shocked to aide in the ability for the plasmid to act as a vector. After allowing the bacteria to reproduce, the antibiotic for which the engineered plasmid conferred resistance will be delivered. All still living bacteria will have acquired the desired traits of both the inserted DNA and the antibiotic immunity. The new proteins or biochemical structures from the inserted DNA can be gathered through different means.

Gene Mutations Using Plasmids

Deletions occur when one or more base pairs are removed from the DNA sequence. A large portion of DNA can be removed from the plasmid by using different restriction endonucleases to cut out a certain segment followed by ligation using DNA ligase to reform a new, smaller plasmid. A single or few base pairs can be removed by using multiple restriction endounucleases that cut near the sticky ends, followed by ligation. Substitutions are a result of the change of a single amino acid in

a protein sequence. This is typically accomplished by changing one (a point mutation) or more base pairs on the genetic code sequence in order to alter the amino acid at a particular site and is known as oligonucleotide-directed mutagenesis. An oligionucleotide is designed such that there is a one base pair difference at a particular site and this one base pair different will encode for a new residue. This oligionucleotide is annealed to the plasmid, which acts as the DNA template, and replication using DNA polymerase results in strands that contain this mutation. One stand of the replicated double helical DNA will be the parent chain and contain the original (wild type) base sequence while the other chain will contain the new (mutant) strand of DNA that encodes for the new desired protein. By expressing the mutant chain, the desired protein can be harvested. Insertions occur when an entire segment of DNA is introduced to a plasmid. The segment of DNA is known as a casette and the technique is termed cassette mutagenesis. Plasmids are cut with restriction enzymes, removing a portion of DNA. Then specifically synthesized or harvested DNA is ligated into that region and the plasmid is expressed and studied.It is also possible to create entirely new proteins and genes by joining together genes that are otherwise unrelated.

Types of Plasmids

Plamids are not required by their host cell for survival. They carry genes that provide a selective advantage to their host, such as resistance to naturally made antibiotics carried by other organisms. Antibiotic resistant genes produced by a plasmid will allow the host bacteria to grow in the presence of competing bacteria that produce these antibiotics. One way to classify plasmids is based on their ability to transfer to additional bacteria. Conjugative plasmids retain tra-genes, which carryout the

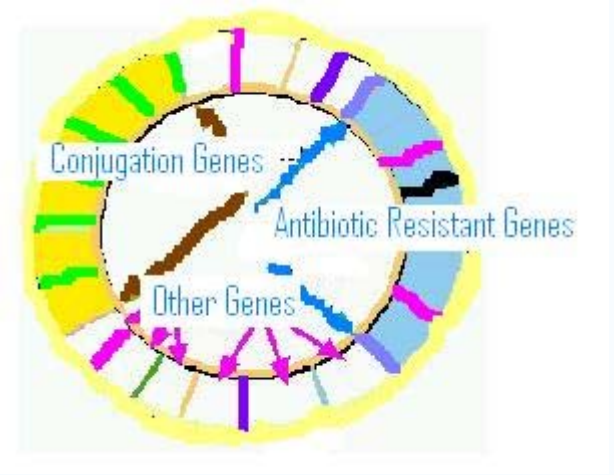
intricate process of conjugation, the transfer of a plasmid to another bacterium. Conversely, non-conjugative plasmids are incapable of commencing conjugation, which consequently can only be transferred via conjugative plasmids. Transitional classes of plasmids are considered to be mobilizable, contain only a subset of the genes necessary for a successful transfer. They have the ability to parasitize a conjugative plasmid by transferring at a high frequency exclusively in the presence of the plasmid. Currently, plasmids are used to manipulate DNA and could potentially be used as devices for curing disease. It is possible for various plasmids to coexist in a single cell. A maximum of seven different plasmids have been found to coexist in a single *E. coli*. It is also possible to find incompatible related plasmids, where only one of the plasmids survive in the cell environment, due to the regulation of important plasmid functions. Hence, plasmids can be designated into groups according to compatibility.

Classification of Plasmids by Function

Another approach to classify plasmids is according to their function. There is a total of five major sub-groups:

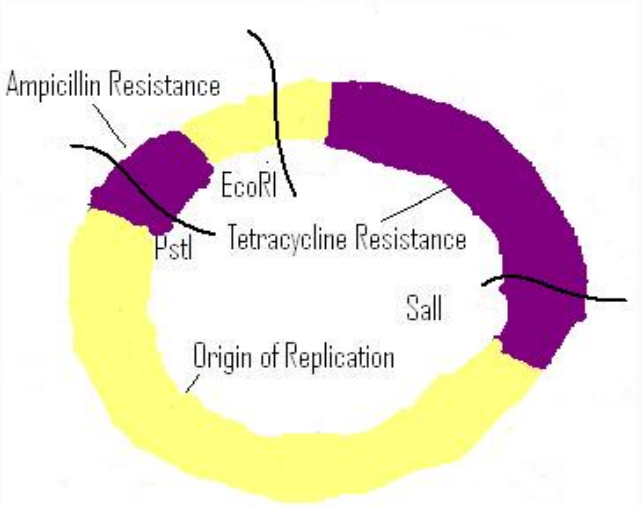
Fertility Plasmids (F-Plasmids)- carry the fertility genes (tra-genes) for conjugation, the transfer of genetic information between two cells. F plasmids are also known as episomes because, they integrate into the host chromosome and promote the transfer of of chromosomal DNA bacterial cells.

Figure 23.Fertility plasmid.



Resistance Plasmids (R-Plasmids)- contain genes that encode resistance to antibiotics or poisons. Examples of R- pBR322 Plasmid. plasmids contains genes for the resistance to tetracycline and ampicillin. Insertion of the DNA at specific restriction sites can inactivate the gene for tetracycline (an effect known as an insertional inactivation) or ampicillin resistance.

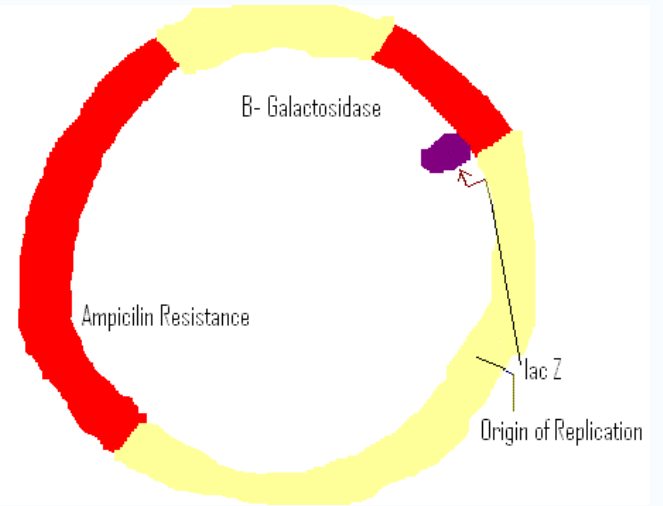
Figure 24. Antibiotic resistant plasmid.



R-plasmid

Plasmid pUC18 has a greater versatility compared to pBR322. Comparable to pBR322, the pUC18 plasmid has an origin of replication and a selectable marker based on ampicillin resistance. Furthermore, this plasmid also contains a gene for beta-galactosidase, an enzyme that degrades certain sugars. while in the presence of a specific substrate analog, this enzyme produces a blue pigment that can be easily detected. Also, this enzyme has been equipped so that it has a polylinker region where many different restriction enzymes or combinations of enzymes can be used to cleave at different locations. Creating a greater variety in the DNA fragments that can be cloned. Interestingly, the insertion of a DNA fragment inactivates the beta-galactosidase. Thus if the blue pigment is not generated, it would be an indication that the DNA fragment was not inserted properly.

Figure 25.

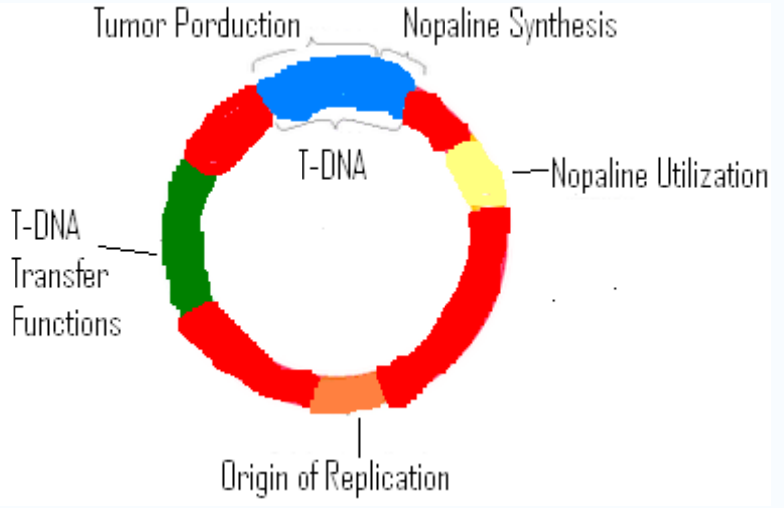


R-plasmid

Tumor Inducing Plasmids (Ti-Plasmids "Virulence Plasmids")- contain *A. tumefaciens*, which carry instructions for bacteria to become a pathogen by

switching to the tumor state and synthesize opines, toxins and other virulence factors. The plasmid effectively transfers foreign genes into certain plant cells.

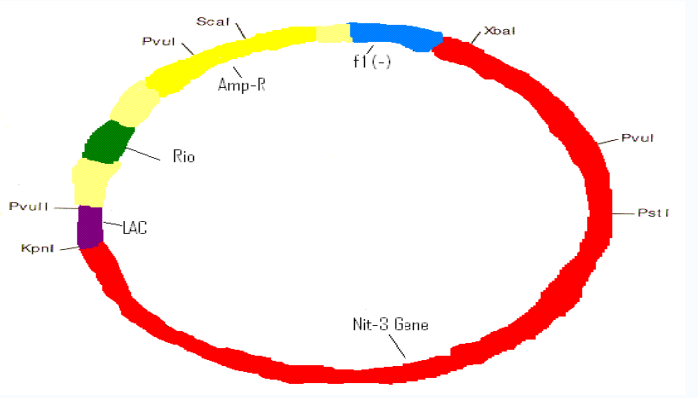
Figure 26. Ti plasmid tumor inducing in plant.



Ti-Plasmid

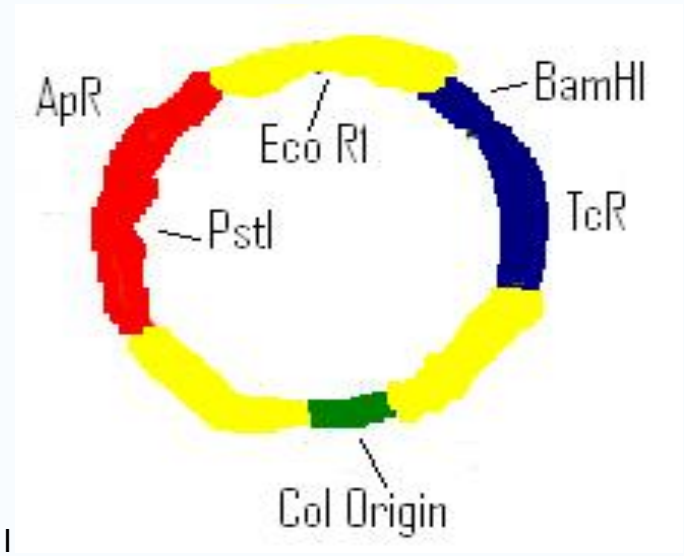
Degradative Plasmids- (Catabolic Plasmid) a type of plasmid that allows the host bacterium to metabolize normally ddifficult or unusual organic compounds such as pesticides.

Figure 27. Degradative plasmid.



Tol- plasmid.

Col- Plasmids- contain genes that encode for the antibacterial polypeptides called bacteriocins, a protein that kills other strains of bacteria. The col proteins of *E. coli* are encoded by proteins such as Col E1 as can been seen in below figure 28.



Col-Plasmid

It is possible for a plasmid to belong to more than one of the above subgroups of plasmids. Those plasmids that exist as only one or a few copies in a bacterium run the risk of being lost to one of the segregating bacteria during cell division. Those single copy plasmids implement systems which actively attempt to distribute a copy to both daughter cells.

Some plasmids include an addiction system, such as a host killing (hok) system of plasmid R1 in *E. coli*. Producing both a long lived poison and a short lived antidote. Those daughter cells that maintain a copy of the plasmid survive, while a daughter

cell that fails to inherit the plasmid dies or suffers a reduced growth rate because of the loitering poison from the parent cell.

Uses, Applications, and Significance of plasmids

Plasmid provides a versatile tool in genetic engineering because of its unique properties as a vector. Plasmids are utilized to create transgenic organisms by introducing new genes into recipient cells. For example, the Ti plasmid from the soil bacterium *Agrobacterium tumefaciens* is very valuable in plant pathology in developing plants with resistance to diseases such as holcus spot on leaves and crown gall tumors. Plasmid also carries medical significance because of its role in antibiotic synthesis. *Streptomyces coelicolor* plasmid can give rise to thousands of antibiotics, as well as that of *S. lividans* or *S. reticuli*. In another example, E. coli plasmids are used to clone the gene of penicillin G acylase, the enzyme that turns penicillin G into the antibacterial 6-amino-penicillanic acid. Once again, these cloning processes are carried out with the assistance of type II restriction enzyme to put the gene of interest into the plasmid vector.

In DNA recombinant technology, plasmid-based reporter gene is crucial as they allow observation of organisms in real time. The gene for Green Fluorescent Protein can be integrated into a plasmid of the organism under investigation. The encoded protein is small and does not alter the function of the host protein. This feature of GFP makes it very easy to observe cell dynamics.

These are only a few among many techniques, applications and uses of plasmids developed throughout the years. The future of plasmid engineering looks very promising with many more examples and opportunities to come.

DNA Microarray

Introduction

In the past, genes and their expression profiles have been studied on an individual basis. More recently technological advances have made it possible to study the expression profiles of thousands of genes simultaneously.

Microarray technology now allows us to look at many genes at once. It allows a quantitative and qualitative comparison between the gene expression patterns of two cells.

What are DNA microarrays

Array means to place in an orderly arrangement. These are also called “DNA chips” or “gene chips” or “biochips” DNA fragment representing a gene is assigned a pecific location on the array.Location of each spot – use to identify a particular gene sequences.30000 spots can be placed in one slide. Principle Based on hybridization probing Uses fluorescently labeled nucleic acid molecules- “mobile probes” Spots are single stranded DNA fragments, strongly attached to the slide.RNA or cDNA is tagged with a fluorescent dye.

Probe - a standardized set of DNA sequences. Target or sample – labeled experimental DNA or RNA. Autoradiography Laser scanning Fluorescence detection devises Enzyme detection system.

Detection methods Hybridization method

Target DNA is labeled and incubated with microarray For the detection of hybridization pattern – reverse dot blot is used Probe high GC content – hybridized more strongly than those with high AT content. matching the target will hybridized more strongly than will probes with mismatches, insertions and deletions Radio

active and non-radioactive methods It involve biotin or digoxigenin labeling require direct detection through, Autoradiography Gas phase ionization Phase ionization Phosphoimagers.

Fluorescence detection method

Multiplexing- one target DNA may be labeled with more than one fluorochromes .

Hybridization can be screened using automatic scanners.

Characteristic features of DNA chips:

PARALLELISM – allows parallel acquisition and analysis of massive data and a meaningful comparison between genes or gene products represented in microarray.

MINIATURIZATION –involves miniaturization of DNA probes and reaction volumes thus reducing time and reagent consumption. Multiplexing –it involve multicolor fluorescence allow comparison of multiple samples in a single DNA chip. Automation –manufacturing technologies permits the mass production of DNA chip and automation leads to proliferation of microarray assays by ensuring their quality, availability and affordability Types of DNA chips Two types- Oligonucleotide based chips cDNA based chips.

Technical application

Green represents control DNA, where either DNA or cDNA derived from normal tissue is hybridized to the target DNA. Red represents Sample DNA, where either DNA or cDNA derived from diseased tissue hybridized to the target DNA. Yellow represents a combination of control and sample DNA, where both hybridized equally to the target DNA. Black represents areas where neither the control nor the sample DNA hybridized to the target DNA.

Spotted microarrays

Probes are oligonucleotides ,cDNA or small fragments of PCR products that correspond to mRNAs and are spotted onto the microarray surface.

Oligonucleotide microarrays

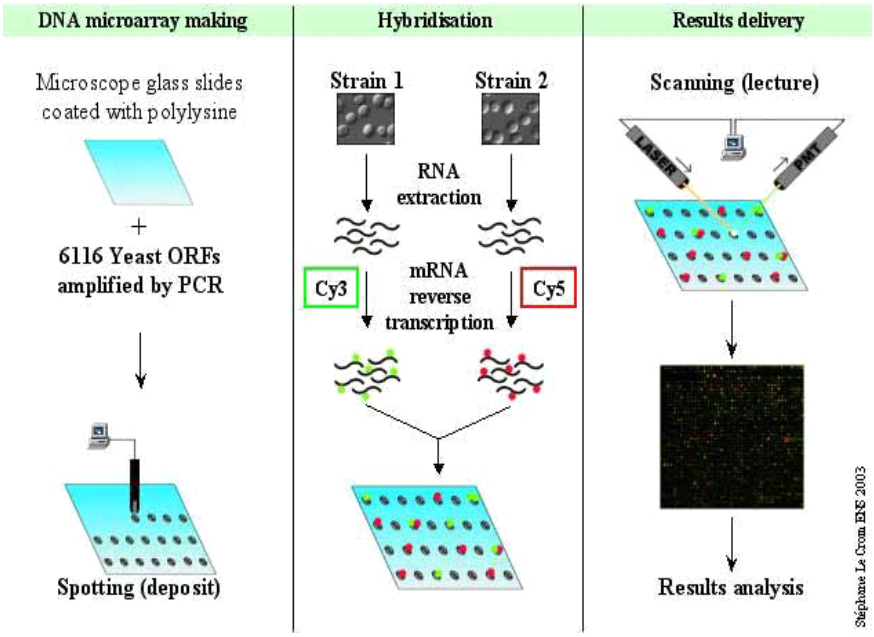
There are commercially available designs that cover complete genomes from companies such as GE Healthcare ,Affymetrix ,Ocimum Biosolutions ,or Agilent.

Figure 29. Micrroarraychips.

Applications of microarrays

Detection of SNPs Characterization of mutant (populations exposed to various selection pressures)Diagnostics and genetic mapping Proteomics.

Figure 30. show DNA microarray principle



Genetic Exchange between Bacteria in the Environment

Introduction

Nucleotide sequence analysis, and more recently whole genome analysis, shows that bacterial evolution has often preceded by horizontal gene flow between different species and genera. In bacteria, gene transfer takes place by transformation, transduction, or conjugation and this review examines the roles of these gene transfer processes, between different bacteria, in a wide variety of ecological niches in the natural environment. This knowledge is necessary for our understanding of plasmid evolution and ecology, as well as for risk assessment of the rise and spread of multiple antibiotic resistance plasmids in medically important bacteria are consequences of intergeneric gene transfer coupled to the selective pressures posed by the increasing use and misuse of antibiotics in medicine and animal feedstuffs. Similarly, the evolution of degradative plasmids is a response to the increasing presence of xenobiotic pollutants in soil and water. Finally, our understanding of the role of horizontal gene transfer in the environment is essential for the evaluation of the possible consequences of the deliberate environmental release of natural or recombinant bacteria for agricultural and bioremediation purposes.

An analysis, based on the acquired, at some time in the distant past, by a differences in base composition and codon utilization variety of pathogens including, *Salmonella phimurium*, *Yersinia pestis,* the extent of this horizontal gene transfer. *Helicobacter pilori*, and variants of *Escherichia coli* . surprisingly, 17.6% of the genes (755 of the 4288 open-reading frames) of *E. coli* have been acquired by horizontal transfer, in 234 events, at a rate of 16 kb/Myr. These are minimal estimates, since events which transferred DNA of similar base composition and codon utilization to that of *E.coli*

would not be detected. In the laboratory, horizontal transfer of genetic material between different bacteria has been detected in a wide variety of different bacterial species and genera. The well-known transfer, by conjugation, of part of the Ti plasmid DNA from *Agrobacterium tumifaciens* to plants and to yeast, demonstrates the horizontal transfer of genes between different phylogenetic kingdoms. The F-plasmid of *E. coli* can similarly effect conjugal DNA transfer to *S. cerevisiae*. Recently, it was shown, under optimized laboratory conditions, that a kanamycin resistance gene integrated in the DNA of a transgenic plant could transform Acinetobacter sp. to Km resistant. Three mechanisms of gene transfer in bacteria have been identified: transformation, involving the uptake and incorporation of naked DNA.

Conjugation, a cell contact-dependent DNA transfer mechanism found to occur in most bacterial genera; and transduction, whereby host DNA is encapsidated into a bacteriophage which acts as the vector for its injection into a recipient cell. These DNA transfer methods have enhanced our understanding of bacterial molecular genetics and have served as elegant tools in the development of genetic engineering technology. By the mid 1980s, biotechnology using recombinant DNA techniques was well developed. The public debate over the hypothetical dangers of the accidental release of genetically manipulated bacteria, and the possibility of horizontal gene transfer to other microorganisms, revealed that we had very little knowledge concerning gene transfer in natural environments. Such knowledge is necessary, in view of the possibility of deliberate release of a variety of nonrecombinant microorganisms into the environment for such agricultural purposes as nitrogen fixation (Rhizobium, Bradyrhizobium, Frankia), phosphate solubilization (Burkholderia, Erwinia), control of phytopathogenic fungi and bacteria (Pseudomonas, Erwinia), plant growth stimulation (Pseudomonas, Azospirillium, Rhizobium, Agrobacterium), insect control *(Bacillus thuringiensis*), weed control (phytopathogenic fungi), bioremediation of xenobiotic-polluted sites (Pseudomonas,

Alcaligenes, Burkholderia, Comamonas), and denitrification (Pseudomonas, Alcaligenes, Comamonas). It was suggested that the properties of the environmentally released microorganisms could be further improved by genetic manipulation. These situations are intrinsically different from those involving the accidental release of an industrial microorganism, for example, *E.coli* designed to produce human growth hormone in an industrial fermentor. The latter case may involve, “tame,” or even disabled, laboratory bacteria, which might be expected to be unable to compete in a natural environment. In contrast, the deliberate environmental application of (natural or recombinant) microorganisms would often have as an objective their stable maintenance and function in a particular environmental niche.

Thus, a rational assessment of the extent of horizontal gene transfer in the environment is needed. Indeed, data are also needed concerning the persistence, survival, competition, nutrition, stress, and physiological state of the introduced bacteria. Such considerations have stimulated the study of the molecular microbial ecology. The present review summarizes recent advances in our knowledge of bacterial gene transfer in a variety of different environmental situations involving plant pathology, rhizosphere microbiology, medical bacteriology, wastewater purification, and bioremediation. No attempt will be made to assess the long-term evolutionary consequences of horizontal gene transfer, nor the potential risks involved in the release of normal or recombinant bacteria in the environment.

The detection of environmental gene transfer

Numerous methods are available for the detection of genetic exchange. Almost all involve the selection for specific genetic characters or phenotypes and this selection imposes a bias on the kind of genes that can be demonstrated to be transferred in situ. For example, genes encoding for the resistance to antibiotics or heavy metals or for the utilization of rare carbon sources (often xenobiotics) are frequently used as selective markers. Such genes are often carried by large self-transmissible plasmids,

or by smaller plasmids that can be mobilized by self-transmissible plasmids. In addition, they are frequently part of transposons or conjugative transposons. Such cases are favorable for detection since plasmids or conjugative transposons may be transferred as a unit and at a high frequency. When such easily selectable phenotypes are available, the genetic transfer experiment may often be performed under natural conditions and the relevant phenotype selected.

More recent work, using green fluorescent protein has removed some of the constraints imposed by the need to select for particular genes. The use of GFP removes the need to cultivate the transconjugants, which has been of concern since it is estimated that, in most ecosystems, less than 1% of bacteria are cultivable using available techniques.

Since gene transfer experiments in the natural environment are technically difficult, most experiments have been performed in microcosms designed to represent the natural environmental situation. Microcosm systems may permit the manipulation of physicochemical variables (temperature, pH, humidity, carbon, nitrogen, and phosphorus sources) that are impossible to manipulate in natural environments. However, microcosms are only an approximation of the natural environment and the results should be viewed within the limitations of their experimental design. In gene transfer experiments, it has been commonly observed that the frequency of transfer is lower in the presence of the native microbial population.

Similarly, a newly introduced bacterial population usually declines upon introduction into the natural environment. The reasons for this may include predation, bacteriophages, growth inhibitors (heavy metals, toxic chemicals, antibiotics, siderophores, bacteriocins), and competition with the resident microflora for nutrients or ecological niche.

The ability to detect gene transfer is dependent on the fate of the transferred DNA once it enters the recipient cell. Many bacteria possess DNA restriction systems which destroy foreign DNA. However, bacteriophages and wide-hostrange plasmids

have evolved ways to counteract these restriction systems by reducing the number of restriction cleavage sites that they contain or by the production of restriction protection systems.

Even when the transferred DNA escapes degradation due to restriction endonucleases, it will not necessarily be passed on to future generations. A plasmid must be capable of replication and maintenance in the new host. If the selected gene is carried by a transposon then the transposon must successfully integrate into the host chromosome or another replicon. Finally, when the fate of the incoming DNA depends upon homologous recombination, as in the case of the conjugal transfer of chromosomal genes or with generalized transduction or transformation, then it must be sufficiently homologous to serve as a substrate for the host recombination system. The efficiency of integration by homologous recombination depends upon the degree of homology of the donor and recipient DNA regions and this is monitored by the mismatch repair system mutS and mutL in *E. coli* .(For example, recombination between *E. coli* and *S. typhimurium* is enhanced 1000 fold when the mismatch repair system is inactivated.

The detection of gene transfer in the environment also depends on the selective advantage or disadvantage that the gene under consideration confers upon the recipient cell. For example, the presence of the Sym plasmid, containing the genes for symbiosis, nitrogen fixation, and nodulation in certain Rhizobia, may confer an advantage for bacteria associated with the rhizosphere of a suitable leguminous host, but offer no advantage, or even a disadvantage, to free living soil bacteria. Similarly, the presence of the genes involved in the degradation of chlorinated aromatic xenobiotics in certain Pseudomonad's may be advantageous or disadvantageous depending on whether that particular xenobiotic is present in the environment and whether that particular bacterium contains the accessory genes necessary for the complete degradation of toxic intermediates. Finally, introduced genes provide only a selective advantage to the recipient if they are expressed.

Many examples of genes that are not transcribed in the new host have been identified. In such cases, the gene expression may occur upon genetic rearrangement, often associated with the presence of a transposon or insertion element.

Transformation

Many species of bacteria are naturally transformable. Some species (e.g., *E.coli*) can be induced to take up DNA by a number of chemical or physical processes including treatment with CaCl2, EDTA, temperature shifts, electro-shocks, and protoplast formation. Recently, natural competence was shown to develop in *E. coli*, at low temperatures in mineral water containing low concentrations (1–2 mM) of CaCl2.

Despite its sensitivity to nucleases, DNA is relatively common in almost all environments and may be excreted by living bacteria or be liberated during autolysis. Environmental DNA can be stabilized by adsorption to sand and clay particles, thereby becoming 100-to 1000-fold more resistant to DNase. Such adsorbed DNA may retain its transforming ability for weeks or even months. The potential dilution of DNA in aqueous environments may seem a barrier to interactions with recipient cells. However, many genetic interactions may take place in a biofilm, rather than between pelagic bacteria.

They estimate that under natural conditions, the lysis of a single cell, in a biofilm, would provide a neighboring cell with significant quantities of DNA that may contribute to horizontal transfer.

Transformation has been demonstrated in different bacteria in a variety of natural ecosystems. Transformation of *Pseudomonas stutzeri*, to rifampicin resistance by chromosomal DNA, in sterile or nonsterile marine sediments. The transformation frequency was lower in nonsterile sediments and in sediments with low organic content. Transformation by a broad-host-range plasmid was similarly detected in a marine Pseudomonas sp. in unamended nonsterile marine-water columns, although addition of nutrients improved the yield. Transformation could take place in

Acinetobacter calcoaceticus growing in biofilms attached to river stones and incubated in natural rivers. The factors affecting transformation of *A. calcoaceticus* in different soil types.

Bacterial Transformation in the Environment

Bacterial host Environmental situation Genetic marker Reference *P. stutzeri* Pseudomonas sp. *A. calcoaceticus A. calcoaceticus A. calcoaceticus A. calcoaceticus E. coli P. stutzeri* ,.

Marine water microcosm. Marine water and sediment microcosm Ground water and soil extract. Ground and aquifer water River epilithon Soil microcosm River and spring water bacteria into oligotrophic soil. Nutrient amendment permitted prolongation of competence and induced competence in cells that could no longer be transformed. Higher phosphate levels also increased the transformation frequency.

The authors note that high nutrient and phosphate levels may occur following the spread of manure slurries on soil. Transformation depended on the soil type, being more efficient in a silt loam than a loamy sand. Soil moisture affected the transformation frequency, with35% soil moisture being optimal. In these experiments, the availability of the DNA for transformation decreased within hours of being introduced in soil.

Transduction

In the process of transduction, bacterial genes are incorporated by bacteriophage particles and transferred to another bacterium. Transduction may be either “generalized” (e.g., by coliphage P1), whereby any bacterial gene may be transferred, or “specialized” (e.g., by coliphage lambda), where only genes located near the site of prophage integration are transferred. Bacteriophages have a restricted host range, sometimes being limited to a single bacterial species.

Furthermore, bacteria may mutate to become resistant (incapable of phage adsorption). For these reasons, transduction would seem an unlikely candidate for

gene transfer in the environment. However, phages are very common in the environment and are relatively stable, being protected by the protein coat. Phages are also more compact and thus more diffusible than naked DNA. Finally, temperate phages may continue to coexist with the bacteria in the form of lysogens and be liberated in some distant future, in response to environmental factors.

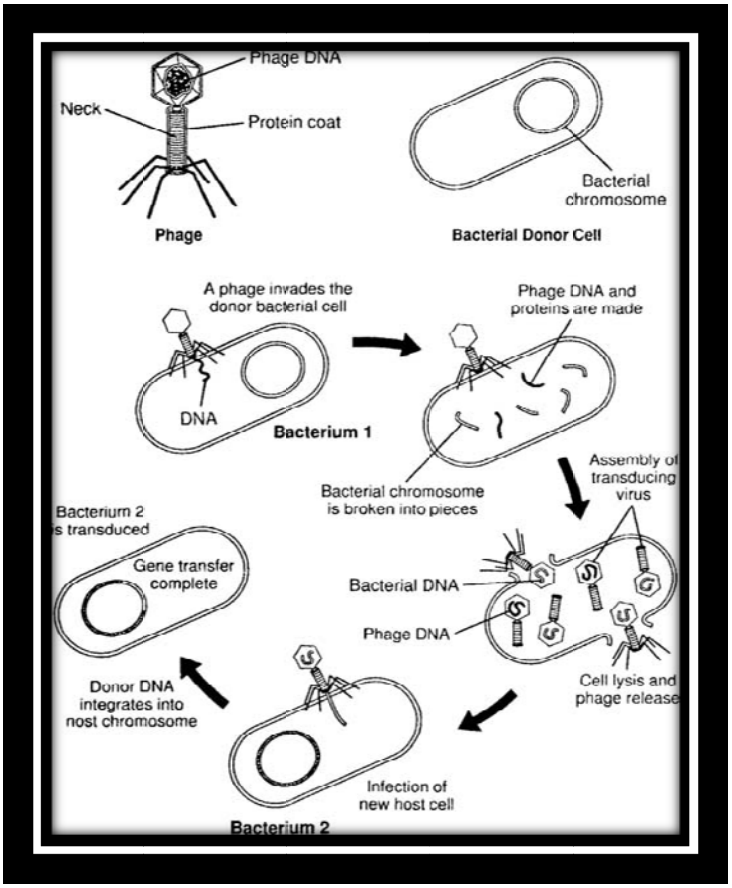
Transduction of both chromosomal and plasmid markers, by *P. aeruginosa* phage F116, was seen in environmental test chambers in a freshwater reservoir. The results suggested that phage liberated spontaneously from a lysogenic strain could productively infect a nonlysogenic host and transduce genes back to the original lysogen. Using the same phage, it was subsequently shown that transduction of plasmid and chromosomal markers of *P. aeruginosa* could take place on the leaf surface. Simulation of field conditions, such as close crop planting, wind conditions, and mechanical disturbances, showed that transduction occurred even when the donor and recipient bacteria were initially present on different plants. A different phage (UT1), isolated from a freshwater habitat, was able to transduce *P. aeruginosa* and also members of the indigenous populations of natural lake-water environments . Recently, a marine phage was shown to facilitate the transduction of a wide-host-range plasmid to members of a natural marine microbial community. Using a mathematical model, the rate of transduction in the Tampa Bay Estuary was estimated at about 1.3 . 1014 events per year. While this calculation involves a variety of assumptions, it nonetheless suggests that transduction may be an important mechanism for horizontal gene transfer in marine environments.

In the field of medical microbiology, some bacteriophages may encode for virulence factors that are expressed upon lysogenization (a phenomenon known as lysogenic conversion).

Bacteriophage-mediated transfer has been suggested to explain the distribution of the bacteriophage-encoded, pyrogenic exotoxin C among different phylogenetic lineages of *Streptococcus pyogenes.* Similarly, phage CTXphi, a relative of

coliphage M13, codes for the cholera toxin of *Vibrio cholera*. Finally, bacteriophages coding for shiga toxin are involved in the pathogenicity of *E. coli* O157:H7 and recent work shows that such phages are common (1– 10/ml) in sewage.

Figure 31. Transduction using phage λ.



Conjugation

The vast majority of reports of bacterial gene transfer in the environment concern conjugation, which may be of several types. (a) Transfer of a self-transmissible conjugative plasmid. The classic examples are the F-plasmid and plasmid RP4 of *E. coli*. (b) Mobilization, whereby non-self-transmissible plasmid, but which nonetheless contains an origin of conjugal transfer oriT, can be transferred by the action of conjugative plasmid (the latter is not usually transferred at the same time). An example is the mobilization of the IncQ plasmid RSF1010 by conjugative IncP1 plasmids such as RP4. (c) Cointegration, whereby two different circular plasmids

may fuse to become one. Thus, a nonself-transmissible nonmobilizable plasmid may nonetheless be sexually transferred due to the action of its cointegrated self-transmissible partner. Such plasmid fusion is often facilitated by the presence, on one of the plasmids, of insertion elements or transposons (for example, of the Tn3 transposon family). Resolution of the cointegrate may occur in the recipient cell.

Chromosomal gene transfer (for example, during Hfr formation by the F-plasmid of *E. coli* is a specialized form of cointegrate formation. (d) Conjugation may also be effected by conjugative transposons which may also facilitate plasmid mobilization and cointegrate formation.

Many plasmids and conjugative transposons are of very wide host range. For example, the nonconjugative, mobilizable IncQ plasmids (e.g., RSF1010) have an extremely broad host spectrum, including most, if not all, gram-negative bacteria and several gram positives such as Streptomyces, Actinomyces, Synechococcus, and Mycobacterium. For this reason, IncQ plasmids have frequently been used as mobilizable cloning vectors . Conjugative plasmids, such as RP4, and conjugative transposons, such as Tn916, are also often of very wide host range.

Members of the Tn916 family are able to propagate in over 50 species of bacteria belonging to 24 different genera. Such transfer systems may have wide evolutionary consequences and have been implicated in the horizontal transfer of antibiotic resistance and xenobiotic degradation genes.

Conjugative Transfer in Gram-Negative Bacteria as a Paradigm for Key Steps in conjugative Plasmid Transfer.

Bacterial conjugation is a highly specific process whereby DNA is transferred from donor to recipient bacteria by a specialized multiprotein complex, termed the conjugation apparatus. An important prerequisite for conjugative transfer is an intimate association between the cell surfaces of the interacting donor and recipient cells. In gram-negative bacteria, this physical contact is established by complex

extracellular filaments, designated sex pili. For the majority of gram-positive bacteria, the means to achieve this intimate cell-cell contact have not yet been identified. To facilitate homology studies with gram-negative systems and to develop a transfer model for gram-positive unicellular bacteria, the current model for conjugative transfer in gram-negative bacteria is briefly presented here. We restrict our overview to the fundamental findings of one of the best-studied conjugative systems, the IncP transfer (tra) system of the broad-host-range plasmid RP4. The IncP transfer system consists of two regions, Tra1 and Tra2, including 30 transfer functions, 20 of which are essential for intraspecies Escherichia coli matings. The central question in bacterial conjugation is how the DNA traverses the cell envelopes of the mating cells. The current model is that two protein complexes exist, namely, the relaxosome and the mating-pair formation (mpf) complex, which are connected via interaction with a TraG-like coupling protein. The relaxosome has been defined as a ultiprotein-DNA complex that is generated at the plasmid origin of transfer, oriT. Plasmid-encoded and chromosomally encoded proteins participate in this complex. The mpf complex is a plasmid-encoded multiprotein complex that is involved in the traffic of the donor DNA strand from the donor to the recipient cell. The RP4 relaxosome was localized in the cytoplasm and found to be associated with the cytoplasmic membrane independent of the membrane-spanning mpf complex. DNA relaxases are the key enzymes in the initiation of conjugative transfer and operate by catalyzing the cleavage of a specific phosphodiester bond in the nic site within oriT in a strand- and site-specific manner. In all systems encoded by self-transmissible and mobilizable plasmids studied so far, the DNA cleavage reaction is a strand transfer reaction involving a covalent DNA-relaxase adduct as an

intermediate. This intermediate is proposed to be a prerequisite for the recircularization of the cleaved plasmid after completion of transfer by a joining reaction between the free 3' hydroxyl and the 5' terminus of the covalently bound relaxase.

An exception is plasmid CloDF13, for which data suggest that nic cleavage possibly results in a free nicked-DNA intermediate.

IncP-type relaxases seem to be the most widely distributed among different gram-positive and gram-negative conjugative plasmids, conjugative transposons, mobilizable elements, and the agrobacterial T-DNA transfer system . All conjugative DNA relaxases have common domains in which the N-terminal moiety seems to contain the catalytic activity whereas the C-terminal moiety may be involved in interactions with other components of the transfer machinery. The enzymatic properties of DNA relaxases are discussed in more detail below.

Biochemical, genetic, and electron microscopic data imply the existence of complicated structures of the mpf complex. Eleven mpf components (trbB to trbL) and traF are required for IncP pilus formation in the absence of any DNA-processing factors, and these components are also required to establish conjugative junctions. The mpf system of RP4 was localized in the cell membrane and was suggested to form a complex that connects the cytoplasmic and the outer membrane. These data agree with a role of the mpf complex in protein transport. Experimental evidence for interaction of the complex with DNA has been recently obtained, since nonspecific DNA binding activity of TrbE was shown.

The tra1-encoded TraG protein is also associated with the cytoplasmic membrane independent of the presence of the Tra2 region. The results also suggest a

connection of TraG with the mpf complex, thereby supporting its proposed role as a potential interface between the mpf system and the relaxosome. Gram-negative bacteria possess two very efficient barriers which have to be traversed by macromolecules during export from and import into the cell: the outer membrane and the inner membrane, which are separated by a cellular compartment, the periplasm. From this point of view, it is evident that macromolecules such as plasmid DNA and prepilin subunits (the building blocks of the pili) need a transport channel to cross the two membranes and the periplasmic space. Conjugative plasmids have evolved systems of regulation that minimize the metabolic and phenotypic load exerted by the maintenance of a conjugative transfer apparatus while optimizing the adaptive advantages of self-transmission. For instance, IncP plasmids transfer at high frequencies under optimal conditions, so that the transfer frequencies can approach one transfer event during a 5-min mating on nutrient agar. However, IncP transfer genes are not expressed constitutively. In fact, their expression is regulated by complex local autoregulatory circuits as well as by global regulators, resulting in the coordinated expression of transfer genes with other plasmidfunctions.

Evidence comes from a wide variety of bacteria in various environmental situations. In some cases it involves the transfer from a known bacterial donor to a a known recipient. However, due to the complexity of the natural ecosystems, it is often the case that the evidence is circumstantial and inferential. For example a plasmid phenotype, such as antibiotic spectrum, restriction pattern, or nucleotide sequence that was previously associated with a particular donor is later found to be associated with a different host. In some experiments. Several gram-positive and -negative

bacterial Human intestine? AR, con-Tn *E. coli* . *E. coli* Simulated sheep rumen AR-P microcosm Human, farm animal, and fish bacterial pathogens) Meat and fish chopping AR-P *B. thuringiensis*. *B. thuringiensis* Lepidopterous larvae Bt-P *Enterobacter cloacae* E. cloacae Cutworm insect gut AR-P E. coli indigenous microflora Soil microarthropod AR , luc-P gut *Erwinia herbicola Enterobacter cloacae* Silkworm larvae AR-P Rhizosphere. *Mesorhizobium loti* Non-symbiotic soil Rhizosphere or soil sym-I *R. leguminosarum R. leguminosarum* Non-rhizosphere soil? sym-*P. putida* and Bush bean leaves cat, gfp-P .leaf surface bacteria Nonpolluted water and soil .

Animal ecosystems

Knowledge of conjugal transfer in the human and animal intestinal tracts is important for understanding epidemics caused by drug-resistant bacteria, and the evolution and origin of multiple drug-resistant transfer factors. Studies have demonstrated that the transfer of antibiotic resistance genes can take place in the intestine between a variety of different gram-positive or gram-negative bacteria.

Direct examination of the nucleotide sequences of resistance genes in different bacteria has clearly confirmed horizontal transfer between bacteria from different habitats. The sequences of the tetM genes from a variety of gram-positive and gram-negative bacteria are virtually the same, suggesting recent horizontal transfer. The tetM gene was found in soil Streptomyces sp. as well as in colonic Peptostreptococcus species, suggesting that soil microbes may transfer genes to intestinal microflora. Similarly, almost identical tetQ genes are shared by Bacteroides sp., which are normal flora of the human gut, the distantly related genus *Prevotella ruminicola*, present in the rumens and intestines of farm animals, and *Prevotella*

intermedia, isolated from the human oral cavity. These studies raise important questions about the transfer of antibiotic resistance genes between the antibiotic-treated farm animals and humans. It is also likely that the normal microflora of the human gut may act as a reservoir of resistance genes which may subsequently be transferred to pathogens. Also disturbing is the fact that the tetQ genes are present on conjugative transposons and that conjugation by these transposons is itself inducible by low levels of tetracycline. Tetracycline is used in animal feed as a growth promoter and in human medicine as treatment for acne and rosaceae, and this may have contributed to the spread of tetracycline resistance over the past 30 years.

Conjugation of multiple drug resistance plasmids, between bacterial pathogens of human, animal, and fish origins and strains from a different ecological niche, was demonstrated in a variety of simulated food-processing environments. Thus, R-plasmids were transferred, in marine water, from the human pathogen *V. cholerae* to the fish pathogen *Aeromonas salmonicida*. Similarly, transfer was observed on a raw salmon cutting board, between a fish pathogen *A. salmonicida* and an *E. coli* strain of human origin. Finally, conjugation was demonstrated in minced meat on a cutting board, between a porcine pathogenic strain of *E. coli* and an *E. coli* strain of human origin.

Plasmid transfer has also been observed in insects. In the digestive tract of the variegated cutworm, *Peridroma saucia*, a low level transfer of antibiotic-resistant plasmid R388::Tn1721 between donor and recipient strains of *Enterobacter cloacae* was observed. In lepidopterous larvae of *Galleria mellonella* and *Spodoptera littoralis*, the efficient transfer of plasmids coding for delta-endotoxin production was observed between different strains of *B. thuringiensis*, suggesting that different insect toxin combinations may be generated in the wild. Similarly, the transfer of

plasmid RSF1010 from *Erwinia herbicola* to *E. cloacae* was detected in the gut of silkworms. Finally, a high level transfer of conjugative and mobilizable plasmids from *E. coli* to a wide variety of strains belonging to the a, b, and . subclasses of the Proteobacteria was demonstrated in the gut of the soil microarthropod *Folsomia candida*. In these experiments the identification of transconjugants was facilitated by the incorporation of the luciferase genes into the plasmids. The gut of *Folsomia candida*, though of small size (10 nl), contains high concentrations of bacteria (1011–1012 CFU/ml) and the resulting cell to cell contact of bacteria, coupled with a nutrient-rich environment, may make it a hot spot for conjugation. metric tons of nitrogen each year.

Water and soil ecosystems

In this review, the transfer of genetic material in uncontaminated and xenobiotic-polluted soil and water environments is treated separately, since the aims of the research and the type of genes studied are usually different in these different ecosystems. Water ecosystems and soil ecosystems, not in direct proximity of the rhizosphere, share the characteristic of being oligotrophic, so that the bacteria are in a state of semi permanent starvation.

Similarly, activated sludge water treatment plants differ from normal aqueous ecosystems in having a large supply of easily assimilable carbon and consequently a large bacterial population. Transfer of plasmids and conjugative transposons between different strains of the intestinal bacterium *Enterococcus faecalis*, in Bavarian municipal sewage treatment microcosm systems. High rates of transfer of sex-hormone plasmids, antibiotic resistance plasmids, and the antibiotic resistance conjugative transposon Tn916 were observed.

Several studies demonstrated the transfer of a HgR plasmid between Pseudomonas strains colonizing either cellulose acetate filters or river stones, that were incubated directly in the river.

There was a linear relationship between log10 gene transfer frequency and the river

water temperature. Biofilm formation on the stones may facilitate cell contact. In subsequent experiments was shown that large conjugative plasmids could be isolated, following conjugation with the indigenous population, in a *P. putida* recipient host containing a mobilizable nonconjugative plasmid. These conjugative plasmids were identified by their ability to mobilize the non-self-transferable plasmid to a suitable target bacterium. Mobilization was also demonstrated on stones in a circulating oligotrophic river water microcosm and occurred even when the donor and the recipient strains were originally on separate stones, showing that simultaneous colonization of new stones by both donor and recipient could occur.

The transfer of the genes coding for resistance to cadmium, cobalt, and zinc from *E. coli* to *Alcaligenes eutrophus*, in non-sterile soil samples, was used to demonstrate that even genes present on Tra. Mob. plasmid may nonetheless be transferred to different genera. This observation raises questions about the biohazard containment properties of Tra. Mob. vectors, which under recombinant DNA containment guidelines had previously been considered relatively safe in connection with the release of genetically engineered microorganisms .

Seawater is an oligotrophic environment containing low levels of assimilable carbon (g/L). Conjugation of plasmid RP4 from *E. coli* to indigenous seawater bacteria could only be demonstrated in the presence of L-brothamended seawater. Using the same plasmid, but with marine Vibrio strains as donor and recipient. conjugation proceeded even when the strains had suffered prolonged starvation (15 days) prior to mating. Mating was still seen when the donor and recipient had been starved for 100 days and 9 days, respectively. Similarly, a bacterial fish pathogen, A. salmonicida, was shown to transfer a marine promiscuous plasmid, pRAS1, to a wide variety of marine sediment bacteria in a microcosm.

One problem with plasmid transfer experiments is that only those transconjugant bacteria that can be cultivated under laboratory conditions will be scored as positive. Indeed only a low proportion of naturally occurring bacteria can be cultivated (the so

called “great plate count.

Plasmid carrying the gene coding for green fluorescent protein. Due to the extreme sensitivity of detection of GFP, the transfer of the plasmid can be monitored in situ at the single cell level. Indeed, as long as the plasmid is transferred and the GFP protein expressed, the stable maintenance of the plasmid is not necessary for the detection of the transconjugant. This may be important since genetic interactions may nonetheless take place between the host bacterium and a transiently maintained plasmid. Using this method, plasmid transfer was detected in bulk seawater and on marine surfaces to a large number of morphologically different bacteria. These results confirm that plasmid transfer and correct synthesis of GFP take place in an oligotrophic environment without addition of exogenous nutrients.

Xenobiotic-contaminated ecosystems

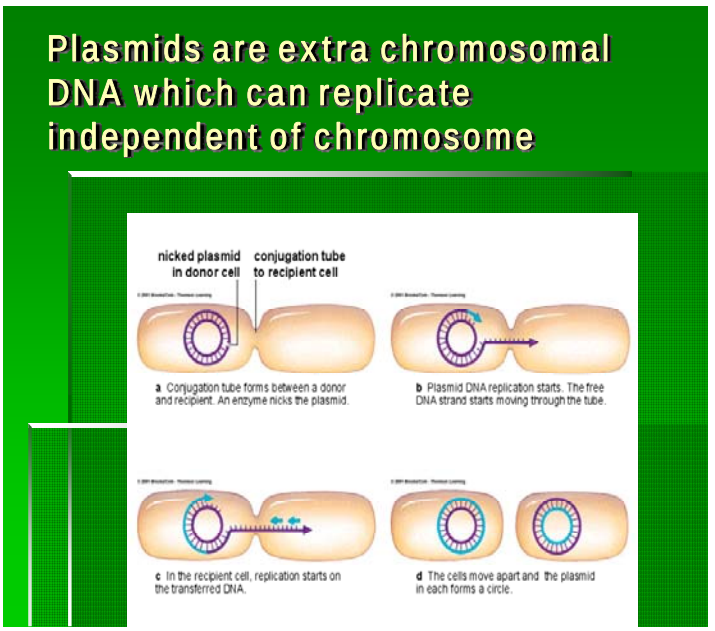
Research in the field of bioremediation concentrates on the idea that the introduction of suitable degradative bacteria may clean up a polluted site more safely and cost efficiently than alternative “burn or bury” methods. Bacteria have been isolated that are able to degrade most man-made pollutants, and most of the degradative genes are part of operons carried by wide-host-range, conjugative, or mobilizable plasmids. It is often observed that the introduced strains are unable to compete with the preadapted indigenous bacteria and disappear without having any effect upon the rate of biodegradation. However, several studies indicate that the plasmids may persist due to transfer to the indigenous population and this may result in improved xenobiotic degradation, the effect of transfer of two different 2,4-D degradative plasmids to the indigenous microflora, on the rate of in situ 2,4-D degradation. The transfer rate depended on a variety of factors such as plasmid type, soil type, indigenous bacterial population, and presence of 2,4-D in the ecosystem.

Genes encoding 2,4-D degradation are typically plasmid borne, but occasionally may be located on the chromosome. Ka and Tiedje (1994) described one strain of

Alcaligenes paradoxux in which the 2,4-D plasmid pKA2 spontaneously integrated into the chromosome and the 2,4-D trait became nontransmissible. The plasmid reappeared after continued culture. A new strain of P. pickettii, isolated in a different soil sample from the same site, was found to contain a plasmid nearly identical to pKA2, indicating horizontal transfer in the field.

It was shown the 2,4-D degradation pathway from a Burkholderia sp. This gene was the first chromosomal tfdA gene to be reported and is only 77% identical to the "classical" tfdA gene from plasmid pJP4. In contrast, it was found to be 99.5% identical to another chromosomal tfdA gene, present in a phylogenetically distinct Burkholderia sp. isolated from a widely separated geographical area. This observation again suggests horizontal chromosomal gene transfer in the environment.

Figure 32. Mechanism of bacterial conjugation.



Quorum Sensing

Introduction

Bacteria use small molecule signals to communicate with each other. Intercellular signaling at high population cell densities is termed quorum sensing and explains many aspects of bacterial physiology observed in single species cultures entering stationary phase in the laboratory. Quorum sensing is used by diverse species to control a multitude of phenotypic traits that often include virulence factors, bacterial signals , exoenzymes and secondary metabolites e.g., antibiotics and biosurfactants In this review, diversity in the biochemistry and molecular biology of signal production, signal sensing, and signal response are discussed. The elucidation of the roles of quorum sensing in bacterial virulence and in biofilm formation will be used to illustrate experimental approaches commonly used. The understanding of quorum sensing obtained in -vitro will be considered in the light of studies describing the activities of bacteria in the real situations of infection and biofilm formation The relevance of quorum sensing to the activities of bacteria in real situations is discussed, taking into account the role of other bacterial species the host on changes in other nonsignalling, parameters within the environment.

Quorum Sensing, Bacterial Signals and Autoinducers

Bacteria are able to sense changes within the environment that they inhabit. On perception of change, bacteria are able to respond by altering their phenotype to provide the activities best suited to success in the new environment. The expression of a modified phenotype often relies on new gene expression. In quorum sensing the environmental parameter being sensed is the number or density of other bacteria particularly of the same species, also present. The study of QS in numerous species has led to the concept of the quorate population, which we can define as a population of bacteria that is above a threshold number or density, and that is able to

coordinate gene expression and, thus, its phenotypic activities.

QS relies on the production and release of small molecule signals by the bacterium into its environment. These signals have also been termed “autoinducers and bacterial “pheromones.” Put simply, the population grows and more signals is produced until a threshold concentration is reached that the bacterium perceives and responds to, by activating (or sometimes repressing) gene expression. The key properties of a QS system are, therefore is the small molecule signal,

The signal synthase

The signal receptor

The signal response regulator

The genes regulated (the QS regulon)

A good example is the control of bioluminescence in symbiotic populations of *Vibrio fischeri* within the light organ of the Hawaiian squid, where only above a certain number of bacteria will be able to produce enough bioluminescence to be visible and assist the squid’s hunting The lux genes are contained within divergent transcripts. The luxR gene transcript encodes a protein housing the signal receptor and the signal response regulator. The transcript of the remaining lux genes luxICDABE of the lux operon is activated by LuxR in the presence of the signal, an acylated homoserine lactone N-3-oxohexanoyll homoserine lactone (3-oxo-C6-HSL).

The signal is produced by LuxI, encoded by the first gene of the lux operon. At low population density, the low level of transcription of the lux operon is insufficient to activate LuxR. As the population grows in the laboratory flask or within the light organ of the Hawaiian squid, the levels of signal reach a threshold level that activates LuxR. The LuxR/3-oxo-C6-HSL complex activates the transcription from the promoter of the lux operon resulting in the following The expression of more LuxI, so more signal is produced and, hence positive feedback occurs. The term “autoinducer” is used by some to describe QS signals because of this positive feedback, whereby the signal induces the production of more signal, for example, the

expression of the luxAB genes that encode the luciferase, luxCDE genes that encode the enzymes that produce substrate for the luciferase and, hence, bioluminescence and the light.

The lux system has been a paradigm for “autoinduction” and QS for many years and the system is now described in great detail. Recent studies have uncovered a greater complexity. One of the most exciting discoveries is that, in addition to the lux operon genes, the QS regulon also contains genes encoding activities involved in the initiation and maintenance of the symbiosis with the squid . Indeed, the ability of bacteria to be able to regulate many genes encoded at different sites on the chromosome with the same system to allow coordination of expression with high cell density is one of the most important features of QS. This is best illustrated in the examples of pathogenic bacteria, in which the regulation of virulence factors, e.g., by *Pseudomonas aeruginosa* or *Staphylococcus aureus* , occurs via QS. A population of significant size can produce sufficient toxins and exoenzymes to overcome a host, whereas lower numbers of bacteria would simply not do enough damage and only induce inflammatory responses that would contain the nascent infection.

The examples mentioned above are based initially on laboratory observations in the culture flask, and sometimes do not wholly reflect the situation in real life In more detailed study, it has been demonstrated that the quorum response may be activated by small numbers of bacteria within a small, enclosed space, e.g., intracellular *S. aureus* in the endosome and that, in some cases, QS may act as a diffusion sensor rather than a sensor of population size. Moreover, in considering QS in the wider environment, it has been demonstrated that other organisms (both prokaryotic and eukaryotic) can perceive respond, and even interfere with the QS activities of a given species in vivo For the purposes of this chapter, it will be assumed that the change in the population parameter is perceived by the bacterium and that the response is a change in gene expression. The nature of signaling mechanisms will be examined first, and then the effect these have on the bacterial phenotype discovery of the

widespread nature of bacteria-to-bacteria signaling has stimulated research that has highlighted the presence of many other potential signal chemistries including unsaturated fatty acids fatty acyl methyl esters quinolones cyclic dipeptides and indole . For some signal structures The small molecule signal defines QS; it is released from the bacterial cell and allows communication with other (bacterial) cells within the population. One significant area for discussion regarding QS has focused on what makes a small molecule found in spent culture supernatants a QS signal? The argument is most intensive around the area of signaling in *Escherichia coli*, because, despite numerous claims of QS roles for various components of culture supernatants, none really satisfy this requirement for QS: that the cellular response extends beyond the physiological changes required to metabolize or detoxify the molecule by Acyl Homoserine Lactones.

Signal generation for acyl homoserine lactones (acyl-HSLs) seems simply to be the coupling of amino acid and fatty acid biosynthesis. Proteins homologous to LuxI represent the major family of acyl-HSL synthases.

However, a second type of acyl-HSL synthase (LuxM family) has been found in Vibrio species. The primary molecular substrates for this reaction have been determined as S-adenosyl methionine (SAM) and acylated acyl carrier protein (ACP) in a number of independent studies for members of the LuxI family X-ray crystallography of LuxI type proteins from *Erwinia stewartii* , 3-oxo-C6-HSL synthase and *P. aeruginosa* N-[3 oxododecanoyl]-l-homoserine lactone [3-oxo-C12-HSL] synthase has been used to explain biochemical and mutational studies of LuxI-type proteins. It is thought that acyl-ACP binds to the enzyme first, which is followed by a conformational rearrangement in the N-terminal region of the protein that precedes SAM binding within an N-terminal pocket containing the conserved residues arginine 23, phenylalanine 27, and tryptophan 33. N-Acetylation of SAM then occurs, followed by lactonisation and the release of acyl-HSL, holo-ACP, and 5¢-methylthioadenosine. The core catalytic fold of EsaI and LasI shares features

essential for phosphopantetheine binding and N-acylation that are found in the GNAT family of N-acetyltransferases and also in LuxM-type acyl-HSL synthases ACP binds to the acyl-HSL synthase at a surface-exposed binding site including residues lysine 150 and arginine 154. Acyl-ACP binding places the acyl group into a hydrophobic pocket (EsaI) or tunnel (LasI). The pocket in EsaI is much smaller than that in LasI, and favours short chain acyl-ACPs whereas the tunnel in LasI can accommodate longer acyl-ACPs.

Both Quorum Sensing EsaI and LasI are LuxI-type proteins that produce -oxo-acyl-HSLs and possess either a serine or a threonine residue at position 140. Acyl-HSL synthases (e.g ,.AhyI, RhlI, SwrI) possessing either alanine or glycine residues at position 140 produce acyl-HSLs lacking C3-substitutions. The side-chain of the amino acid at position 140 protrudes into the acyl-chain pocket and mutation of EsaI to valine at 140 reduces enzyme activity, presumably by reducing access to the pocket. Mutation of EsaI to alanine at 140 shifts the preference of the enzyme to acyl-ACP substrates without a C3-substitution Advances in understanding the mechanisms of synthesis and acyl side chain specificity will be of benefit in designing novel antipathogenic drugs that may prevent activation of virulence gene expression by inhibiting acyl-HSL synthesis Posttranslationally Modified Peptides.

For peptide signals, the ribosomal synthesis of a precursor propeptide is followed by processing, which often introduces other chemical groups such as lipid moieties as with the ComX pheromone of *Bacillus subtilis* or intramolecular bonds such as thiolactone, in the staphylococcal auto inducing peptide. Then, a cleavage of the processed precursor occurs to release the mature peptide AI-2: The LuxS Signal To date, the only QS system shared by both Gram-positive and Gram-negative organisms involves the production of AI-2 via LuxS (Surette et al. 1999; Xavier and . In *Vibrio harveyi,* the regulation of bioluminescence is under the control of parallel QS systems . System 1 involves an acyl-HSL synthesised by a LuxM synthase, and the LuxN receptor kinase sensor. In System 2, the signal synthase is LuxS and the

signal (AI-2) is a furanosyl borate diester (3A-methyl-5,6-dihydro-furo -٢,٣]D][1,3,2] dioxaborole-2,2,6,6A-tetrol; abbreviated as S-THMF-borate) as identified from X-ray crystallography of the ligand-bound receptor, The luxS gene is conserved in many bacterial species and molecules activating an AI-2 biosensor are found in spent supernatants from diverse bacterial species, including both Gram-positive and Gram-negative bacteria and leading to the suggestion that AI-2 may be a universal signal for interspecies.

AI-2 is formed as a metabolic byproduct of the activated methyl cycle (AMC). The AMC recycles SAM, which acts as the main methyl donor in eubacterial , archaebacterial, and eukaryotic cells. After methyl donation, SAM is converted to a toxic metabolite S-adenosyl-l-homocysteine SAH). Detoxification of SAH in *V. harveyi, E. coli*, and many other bacteria is a two-step process, involving first Pfs enzyme (5¢-methylthioadenosine/S-adenosylhomocysteine nucleosidase) to generate S-ribosyl homocysteine (SRH), which acts as the substrate for LuxS. SRH is converted to adenine, homocysteine (which is converted to methionine and then SAM), and DPD, the precursor for AI-2. Some bacteria and eukaryotes are able to replace this two-step reaction with a single enzyme, SAH hydrolase, which converts SAH to homocysteine without producing AI-2. The DPD precursor is a highly unstable molecule that may spontaneously interconvert to a number of related structures depending on the environment Waters and including the form that is stabilised by forming a complex with boron in AI-2 signaling in *V. harveyi* system 2. The putative AI-2 signals of other bacteria, e.g., E. coli, may be formed via different routes depending on the cyclisation product of DPD . It is hypothesized that alternate forms of AI-2 may be more active within a specific niche or may reflect the variation in the function of AI-2, such as QS versus metabolic roles.

Is Signal Generation a Regulatory Step ?

In many cases, the expression of signal synthase forms part of the quorum response providing positive feedback that allows a rapid induction of the high cell density

phenotype (e.g., *V. fischeri*). For some signals, substrate availability may coordinate signal production with nutrition, although there is little evidence to suggest that this is a widespread strategy..

How does the Signal exit the cell ?

In the case of acyl-HSL molecules with short acyl chains, the freely diffusible nature of these molecules has been demonstrated . Acyl-HSLs with longer acyl chains do not seem to escape the cell membranes as easily, and 3-oxo-C12-HSL, for example, is actively pumped from the P. aeruginosa cell Peptide signals commonly undergo active export, with ATP-binding cassette (ABC) transporters commonly used (e.g for CSP[competence-stimulating peptide, *Streptococcus pneumoniae*], CSF]competence- and sporulation-stimulating factor, also termed the Phr pheromones which is Sec dependent; Note the PhrA signals controlling sporulation in Bacillus are thought to be part of an export–import circuit in which signals are exported from the bacterial cell, undergo processing, and are then reimported via the oligopeptide permease (Opp) system. It is thought that only the producer cell is affected and that these pheromones are not a population-wide signal.

Signal perception and response regulation

In QS, the environmental parameter the bacterium perceives is the level of signal external to the cell. Perception of the signal can be accomplished by surface exposed membrane receptors or intracellular receptors . For the major classes of signal acyl-HSLs, AI-2 and posttranslationally modified peptides, examples of both internal and external sensing are apparent .

The response to signal perception is intracellular, most commonly affecting activation or repression of gene expression. In the simplest case, the signal diffuses into a cell and acts as a ligand for a protein influencing the initiation of transcription for extracellular perception, signal transduction via phosphotransfer to proteins affecting transcription occurs LuxR Receptors for Acyl-HSLs.

Perception of acyl-HSLs by LuxR family response regulators is intracellular.The

LuxR-type acyl-HSL receptors can be described as an N-terminal acyl-HSL binding domain and a C-terminal transcriptional regulatory domain that contains a helix-turn-helix (HTH) DNA binding motif. Interaction with DNA is as a dimer, recognizing a sequence of dyad symmetry located within the regulatory region of target genes. The recognition sequence, a lux or lux-type box, is approximately ٢٠ bp in length.

The majority of LuxR-type proteins studied in detail to date are transcriptional activators, when bound to their co activating acyl-HSL ligand. TraR (Agrobacterium *tumefaciens*), LuxR (*V. fischeri*), and LasR and RhlR (both *P. aeruginosa*) bind to their recognition sequences as dimers, or higher-order multimers in the case of CarR (*Erwinia carotovora* subsp. carotovora [Ecc]) and the recruitment of RNA polymerase at the target promoter .

The LuxRtype proteins bind their acyl-HSL ligands in a 1:1 stoichiometric ratio. In the case of *A. tumefaciens* ,TraR perceives the N-(3-oxooctanoyl)-l-homoserine lactone (3-oxo-C8-HSL) signal as a monomer on the inner face of the inner cytoplasmic membrane. Holo-TraR dimerises and is cytoplasmic, where it acts as a transcriptional activator for the quorum response. Not all LuxR-type proteins act as transcriptional activators. Genetic, in vitro DNA binding assays and phylogenetic studies have identified EsaR (*Erwinia] stewartii;YpsR (Yersinia paratuberculosis, SpnR (Serratia marcescensExpR Erwinia chrysanthemi* and VirR as a group of LuxR-type proteins that act as repressors in the absence of their derepressing cognate acyl-HSL .

X-ray crystallography has revealed that LuxR-type proteins interact with their ligand at an acyl-HSL binding cavity . The highly conserved residues at position 57 (tryptophan) and 70) aspartate are important in the stabilisation of acyl-HSL binding. Mutations in TraR in this region have identified the tyrosine at position 53 as being important in discriminating in favour of the 3-oxo substituted ligand .Other mutations in the acyl-HSL cavity of LuxR-type proteins have affected chain length specificity. Studies with various analogues of the acyl-HSL signal have identified a number of

agonistic and antagonistic structures. The most important conserved feature of the signal that affects its activity as a ligand is chain length, but various alterations of the lactone ring head group have also been shown to have profound effects Using studies of LuxR and TraR as evidence it is thought that ligand binding induces a conformational change in the LuxR-type transcriptional activators that permits dimerisation and unmasks the DNA binding domain. After DNA binding, there is also evidence to suggest that interaction with the C-terminal domain of the alpha subunit of RNA polymerase contributes to the recruitment of RNA polymerase, and to the initiation of transcription . In studies of repressor LuxR-type proteins, it seems that the apo-protein binds DNA and blocks access to the promoter.The presence of the appropriate ligand releases the repression, and it is hypothesized that ligand binding induces conformational changes that interfere with DNA binding .LuxN-Type Receptors for Acyl-HSLs.

The investigation of the control of bioluminescence in *V. harveyi* and *V. fischeri* has identified not only a second acyl-HSL synthase family, but also a membrane receptor family. In *V. harveyi*, N-(3-hydroxybutanoyl)-l-homoserine lactone (3-hydroxy-C4 -HSL) is produced by LuxM . In the absence of signal, the sensor kinase LuxN autophosphorylates and relays phosphates to LuxU a regulator also phosphorylated by two other sensor kinases, LuxQ and CqsS.

LuxQ is the sensor kinase perceiving AI-2 LuxU phosphorylates LuxO, which activates the expression of a collection of small regulatory RNAs (sRNAs) at .54-dependent promoters.

In the presence of the RNA chaperone, Hfq, the sRNAs destabilize the mRNA encoding LuxR . LuxR here is a transcriptional activator for the luxCDABEGH operon and other genes involved in virulence, but not an acyl-HSL receptor and not homologous to *V. fischeri* LuxR (In the absence of functional LuxR, there is no bioluminescence).

At high cell density, 3-hydroxy-C4-HSL is produced (and also the ligands activating

LuxQ and CqsS), inducing LuxN, LuxQ, and CqsS phosphatase activities that dephosphorylate LuxU and lead to the inactivation of LuxO, allowing LuxR to be expressed and transcriptional activation to occur (Homologues of LuxM and N have been found in *V. fischeri* (AinS and R) and *V. anguillarum* (VanM and N), where they contribute to the regulation of gene expression through a phosphotransfer pathway involving LuxU and LuxO-type proteins.

The Response to AI-2 in *V. harveyi*, AI-2 (S-THMF-borate) is bound by the periplasmic protein LuxP, which then activates the dephosphorylase activity of LuxQ, leading to inactivation of LuxO and the expression of LuxR. LuxP and Q homologues exist in other vibrio species, where they are involved in the control of virulence factor expression (*V. cholerae, V. anguillarum, and V. vulnificus*) bioluminescence and symbiosis factors (*V. fischeri*). Molecules able to activate LuxQ are produced by many other bacteria via LuxS and are also termed AI-2. There is debate regarding whether these molecules are actually QS signals, or whether they are simply waste products of the AMC Certainly, luxS mutations have profound phenotypic effects, but these may be caused by the toxic effects of disrupting the AMC. One question is whether these other bacteria possess AI-2 receptors and signal transduction mechanisms to affect gene expression. Although there are homologues of LuxP, Q, U, and O; they are only found together in *Vibrio* species . Unlike AI-2 signaling in these Vibrio species where a phosphorylation cascade is initiated when extracellular threshold levels of AI-2 are reached, AI-2 signaling within *E. coli,* Salmonella, and other organisms depends on the active uptake of DPD. In Salmonella, the cyclic derivative of DPD, (2R,4 S)-2-methyl-2,3,3,4-tetrahydroxytetrhydrofuran (R-THMF), binds to a homologue of the periplasmic binding protein LsrB. LsrB is part of an ABC transporter encoded by the lsrACDBFGE operon. The putative ATPase of the ABC transporter, a sugar binding protein, a membrane channel, and other proteins encoded by the lsr operon show similarity to proteins encoded by the b1513 operon in *E. coli.* The repressor LsrR

regulates the lsr operon. Downstream of lsrR is a gene encoding an AI-2 kinase. Phosphorylation of AI-2 is proposed to occur after import to allow sequestration within the cytoplasm. Phosphorylation of AI-2 causes LsrR to relieve its repression of the lsr operon, allowing further AI-2 import the Response to Modified Peptide Signals.

Two-component signal transduction systems predominate in Gram-positive bacteria . The majority of peptide signals are perceived by sensor kinase proteins, which generally activate transcriptional activators of the quorum response. The posttranslationally modified peptide (e.g., AIP in *S. aureus* ,ComX in *B. subtilis*) binds to the surface-exposed transmembrane receptor histidine kinase (e.g., AgrC in *S. aureus*; ComP in *B. subtilis*), promoting autophosphorylation .

Phosphotransfer to the response regulator (e.g., AgrA in S*. aureus;* ComA in *B. subtilis* initiates expression of the quorum response. In S. aureus, two promoters are activated in the QS regulon: the agr promoter P2 activates expression of RNAII, which encodes agrBDCA; and the agr promoter P3, which encodes the regulatory RNA, RNAIII . Phospho-AgrA, and AgrA, to a lesser extent, bind to consensus DNA sequences for the LytR family of response regulators Phospho-AgrA binds to the P2 site with approximately a 10-fold greater affinity than to the P3 site. In vitro electrophoretic mobility shift assays with wild-type and mutant P2 and P3 sequences demonstrated that a deviation of two bases in P3 away from the consensus LytR sequence was responsible for the differential binding is proposed that AgrA first activates the P2 promoter, where autoinduction initiates positive feedback that increases AgrA concentrations to activate transcription at P3. AgrA activates transcription from P2 and P3 in concert with another global regulator SarA, that has been shown to bind agr promoter DNA. SarA and AgrA DNA binding footprints overlap on P2, and the details of how these two regulators interact to control expression are subject to speculation. Phospho-ComA directly activates transcription at a number of promoters, and a palindromic consensus binding site

sequence has been identified . In addition to the directly activated genes of the ComX quorum response in *B. subtilis*, there is indirect activation of more than 100 genes through the activity of competence transcription factor, ComK and the expression of an additional 89 genes is indirectly affected through ComK-independent In Gram-positive bacteria, the exception to the two component signal transduction system rule are the Phr pheromones of *B. subtilis* that enter the cell through the OPP Phr pheromones are perceived internally by Rap phosphatases (which they inhibit) and, thereby, influence the quorum response by affecting the level of phosphorylated transcriptional activators.

The Quorum Response and the QS Regulon

In the first studies of QS, the phenotypic traits under investigation were known, and it was their regulation that was under investigation (e.g., the control of bioluminescence in *V. fischeri*). In later studies, the signaling mechanism was identified first and then the extent of the regulon was determined. A strategy of mutation of the signaling genes and observation of high cell density phenotypic traits was developed to identify regulated genes and contributions to whole phenotypes, e.g., to biofilm formation or virulence . The analysis of signaling mutants, using both proteomic and transcriptomic approaches, is now being applied to further describe the quorum response.

It is now clear that the quorum response is comprised of directly controlled genes the QS regulon and indirectly controlled genes. Direct control of transcription by QS activates, or, in some cases, derepresses, gene expression. The model is simple signal accumulates, acts to stimulate DNA binding by a transcriptional activator or reduces DNA binding by a repressor, and new gene expression occurs at genes that are at least open for transcription (i.e., not being repressed by a second mechanism).

In *Agrobacterium tumefaciens*, a process of anti-activation can occur, in which TraM can form a stable complex with TraR/3-oxo-C8-HSL and which can even disrupt the

TraR–DNA complex to ensure that activation of the quorum response occurs at the correct time Regulatory proteins and sRNAs are also part of the QS regulon and these mediate indirect QS effects on the quorum response. The contribution of these secondary regulators of the quorum response is especially apparent in DNA microarray studies analyzing the bacterial transcriptome.

QS is not the only factor controlling gene expression, and other inputs are essential in controlling what we define as the quorum response. Some genes will not be expressed unless cell density and another environmental parameter are satisfied The clearest illustration of this came from a comparison of what happens when the cognate signal is added exogenously to *V. fischeri, Erwinia carotovora*, and *P. aeruginosa* .

In *V. fischeri*, the expression of bioluminescence is advanced, and expression may occur at low cell density. The same is true for carbapenem biosynthesis in *E. carotovora* , but it is not possible to advance ,for example, exoenzyme production by *P. aeruginosa* without first making mutations in additional regulators.

The early studies of QS developed from investigations of particular phenotypic traits i.e., bioluminescence of *V. fischeri* carbapenem biosynthesis by *Erwinia carotovora*, elastase production by *P. aeruginosa* and conjugation in *Agrobacterium tumefaciens* and their regulation. As the importance of this novel regulatory mechanism became apparent, similar systems were sought in other species and there was renewed interest in other signaling systems including the regulation of conjugation in *Enterococcus faecalis* and the production of antibiotics by Streptomyces . Initially, this was particularly fruitful ,with reporter strains used to demonstrate signal production and screen for signal synthase clones or null mutants. More challenging has been the search for true QS signaling systems in bacteria such as *E. coli*, in which many molecules have been identified from culture supernatants that influence gene expression .

QS in E. coli

Salmonella and *E. coli* do not have a luxI gene or any acyl-HSL synthase and, therefore ,do not synthesize acyl-HSLs. E. coli and Salmonella do possess a LuxR-type protein ,SdiA, which is acyl-HSL responsive and regulates genes contributing to the adhesion to host tissues and the resistance to complement killing The biological role of SdiA, and the detection of acyl-HSLs presumably produced by other bacterial species, is yet to be defined .E. coli and Salmonella are paradigm species of bacterial life and it is hypothesized that they must surely produce a QS signal. For this reason, the culture supernatants of *E. coli* and Salmonella have been extensively interrogated for the presence of potential QS signals, and many candidates have been proposed) The role of AI-2 as a QS molecule in *E. coli* and Salmonella is controversial. In the true sense of the word, a cell-to-cell signalling molecule is a small diffusible molecule that has a function in cell-to-cell communication Within conditioned media, a large number of bacterial products can be found and may have the potential to serve as cell-to-cell signals within a QS system. The presence of bacterial products, e.g., fermentation metabolites and medium degradation products all provide a milieu that, when added to culture of low cell density, will trigger a variety of responses unrelated to cell-to-cell signaling.

Conversely, a true signaling molecule is produced during specific stages of growth, under certain physiological conditions or in response to environmental change. The molecule accumulates extracellularly and is recognised by a specific receptor. Threshold concentrations of the molecule generate a concerted response in which the cellular response extends beyond physiological changes required to metabolize or detoxify the signaling molecule. Is AI-2 an *E. coli* or Salmonella QS Signal ? Studies comparing luxS mutants (unable to produce AI-2) with wild-type *E. coli* found that, based on DNA microarray analysis, greater than 400 genes were either up regulated or down regulated in the luxS mutant when compared with the parent strain, concluding that AI-2 signaling was a global regulatory system in *E. coli.* This study neglected the fact that LuxS was vital for the AMC and the production of a

feedback mechanism within the cycle (via SAH). The luxS and pfs genes are located adjacent to other genes involved in metabolic reactions linked to the AMC, further suggesting a role in metabolism rather than QS. There is strong argument for the LuxS protein as a metabolic enzyme involved primarily in the detoxification of SAH, and AI-2 is a byproduct of this process.

The regulation of type III secreted virulence determinants, flagella, and motility genes has been linked to AI-2 signaling in enterohaemorrhagic *E. coli* (EHEC) Careful study of this regulation has demonstrated a regulatory role for another extracellular product, AI-3, that is not produced by a luxS mutant. AI-3 is the activating signal for virulence gene transcription and is not dependent on LuxS for synthesis. It is proposed that the pleiotropic effects of a luxS mutation on AMC and amino acid metabolism affects the availability of synthesis precursors for AI-3 .AI-3 is a chemically distinct molecule from AI-2 in that it binds C-18 HPLC columns and can only be eluted with methanol .

AI-2 and AI-3 activity may be differentiated by two assays. AI-2 produces bioluminescence in *V. harveyi*, whereas AI-3 shows no activity and AI-3 is able to activate transcription of virulence genes in EHEC in which AI-2 has no effect. The catecholamine neurotransmitters epinephrine and nor epinephrine can replace AI-3 as a signal in the regulation of virulence genes in EHEC ,here these effects may also be blocked by adrenergic receptor antagonists, suggesting that AI-3 may be structurally similar to epinephrine and norepinephrine and have a role in host–bacteria communication.

A membrane sensor kinase, QseC, is activated by AI-3, epinephrine, and norepinephrine ,suggesting a role in intraspecies, interspecies, and interkingdom signaling. QseC is part of a two-component system as a sensor kinase activating response regulator QseB to activate transcription of the flagella regulon for swimming motility in EHEC. Amino acid sequence analysis shows that QseC is conserved in other enteric bacteria that have also been shown to respond to

catecholamines, e.g., Shigella, Salmonella, and Yersinia. AI-3-, epinephrine-, and norepinephrine-activated QseC also activates another response regulator, QseA , which is one of many activators of the expression of the genes encoded on the locus of enterocyte effacement (LEE) locus of enteropathogenic *E. coli* EPECand EHEC, and, therefore, central to the regulation of enterovirulence in these

Pathogens. In *E. coli*, it is clear that extracellular products can affect gene expression and hence, bacterial phenotype. In the case of AI-2, and also other molecules such as indole (Wang et al. 2001), it is likely that the phenotypic changes observed were consequences of experiments that disrupted normal metabolism. The evidence supporting a role for AI-3 as a signal molecule is stronger, although whether that is as a true QS signal or possibly as an amplifier of host signals, e.g., catecholamines produced by damaged tissue, is an issue yet to be resolved.

Regulation of Microbial Physiology by QS

QS controls gene expression and defines a high cell density phenotype. Research studying the various components of the high cell density phenotype has identified some common traits, regulated by QS in its various evolutionary forms. That is to say that whether bacteria use acyl-HSLs, modified peptides, activators, or repressors to actuate their QS control, there are a number of traits that seem to be commonly regulated by QS. To illustrate this, the example of the regulation of biosurfactant secondary metabolites will be discussed. In many cases, QS coordinates the activation (or repression) of transcription from numerous promoters at sites on the bacterial of expressed gene the QS regulon. It is clear here that the coordinated combination cts is necessary for the bacterial population to display phenotypes that are more complex. The roles of QS in the control of virulence and biofilm formation will be discussed as examples.

QS and Secondary Metabolism

A secondary metabolite is a compound that is not necessary for growth or maintenance of cellular functions but is synthesised, often for the protection of a cell,

during the stationary phase of the growth .

Microbial biosurfactants are surface-active molecules produced by a wide variety of microorganisms, including bacteria, yeasts, and filamentous fungi. The surfactant properties of these molecules may be attributed to their amphipathic nature in that they are composed of both hydrophobic and hydrophilic moieties .

This enables them to effectively reduce surface and interfacial tensions, dissolve hydrophobic compounds, and alter the hydrophobicity of the microbial cell surface .

The phylogenetic diversity of organisms that produce biosurfactants is reflected in their varied chemical structures and surface properties. All known microbial biosurfactants are classified as low molecular weight, high molecular weight, or particulate biosurfactants (Desai and Banat 1997). The hydrophilic component is usually an amino acid, polypeptide, monosaccharide, disaccharide ,or polysaccharide, and the hydrophobic component is usually a saturated or unsaturated fatty acid. Low molecular weight biosurfactants are glycolipids ; lipopeptides or lipoproteins; and fatty acids, phospholipids and neutral lipids.

The synthesis and regulation of biosurfactant production is directed by specific environmental signals and is often a cell density-dependent phenomenon. The diversity of chemical structures and physicochemical properties of biosurfactants indicates that they are synthesized by microorganism for a variety of purposes. These include .Enhancing the bioavailability of hydrophobic substrates by forming micelles/emulsions.

To facilitate the surface translocation of swarming bacteria by overcoming surface tension. Attachment and detachment of bacteria from hydrophobic substrates by influencing cell surface properties .

Growth of bacteria on hydrophobic substrates such as polyaromatic hydrocarbons (PAHs) stimulates the bacterial synthesis of biosurfactants, so as to facilitate the use of these compounds as a source of carbon. Because growth on such substrates is limited to the interface between water and oil, the release of biosurfactants enhances

bacterial growth by partitioning at the hydrophobic–hydrophilic interface .

This increases the surface area over which the bacteria can grow. It is mostly at this interface that bacteria can proceed to degrade the compound, with the help of their surface-associated oxygenase enzymes that oxidise the highly reduced ring structures that characterise hydrophobic xenobiotics. The key, therefore, to more efficient, accelerated growth on hydrocarbons is increased contact between cells and hydrocarbon, and this is afforded by the biosurfactants. From a bioremediation perspective, this is crucial because the initial ring cleavage is the rate-limiting step in biodegradation, and microbial biosurfactants can overcome this limitation.

A second environmental advantage of biosurfactant production is swarming migration in bacteria. Bacterial swarming is a flagella-driven movement accompanied by the production of extracellular slime, including biosurfactants. Swarming may be considered as a means to colonize new niches that are more nutritionally endowed (reviewed by Daniels et al. 2003). It is cell density dependent, with specific nutritional and surface associated signals that lead to differentiation of cells into the swarmer state. Biosurfactants function as wetting agents by reducing the surface tension, thus, facilitating the smooth movement of these cells. Mutants deficient in biosurfactant production are unable to spread over a solid surface such as an agar plate.

Biosurfactants are known to alter the surface properties of the secreting cell, which may, in turn, influence the interaction between the cell and the hydrocarbon.Cell surface properties arise from the unique chemical structure of the cell surface .For example, the Gram-negative bacterium, P. aeruginosa, has an outer membrane containing lipopolysaccharides (LPS). The variable O-Antigen of the LPS extends into the surrounding environment and consists of 15 to 20 repeating monomers of a three- to five-sugar subunit. The structure of this O-Antigen contributes to cell surface hydrophilicity. The interaction between the surfactant and the bacterial cell is thought to occur in two ways. .Formation of micelles that coat the hydrophobic

compound and, thus, allow its uptake into the cell. .Altering the cell surface hydrophobicity by the release of LPS.

In the second instance, the biosurfactant may interact with the cell surface in two ways to cause changes to its hydrophobicity. The biosurfactant rhamnolipid directly removes the LPS through its solubilization or indirectly through the complexation of magnesium cations that are crucial for maintaining strong LPS–LPS interactions in the outer membrane.

In either case, the loss of the LPS from the outer membrane results in high adherence to hydrocarbons and enhanced degradation of the hydrophobic compound .Therefore, biosurfactants interact with the secreting cells to determine the outcome of the cells' interaction with its environment.

Rhamnolipid Production by *P. aeruginosa*

Rhamnolipids are classified as low molecular weight glycolipids, composed of disaccharides acylated with long-chain fatty acids or hydroxy fatty acids. They are mainly produced during growth on hydrocarbons or carbohydrates. Their synthesis occurs at late exponential or stationary phase and is usually associated with nitrogen limitation. Rhamnolipids consist of one or two molecules of the sugar rhamnose linked to one to two molecules of .-hydroxydecanoic acid .Various types of rhamnolipids have been identified depending on the combinations of rhamnose and decanoate. The rhamnolipids principally detected in culture supernatants include rhamnolipid (l-rhamnosyl-l-rhamnosyl-.-hydrocydecanoyl-hydroxydecanoate) and rhamnolipid 2 (l-rhamnosyl-.-hydrocydecanoyl -hydroxydecanoate. Rhamnolipid is produced during the stationary phase of growth, and biosynthesis occurs via a series of glycosyl transfer reactions catalysed at each step by specific rhamnosyltransferases (Ochsner and Reiser 1995). The nucleotide-linked sugar thymidine diphosphate-rhamnose (TDP-rhamnose) is the donor and .-hydroxydecanoyl -.hydroxydecanoate is the acceptor. Rhamnosyltransferase 1 (catalysing the first step in rhamnolipid synthesis) is encoded by the rhlAB operon

(Ochsner et al 1994) which is able to restore rhamnolipid activity in mutant strains The operon encodes two proteins, RhlA and RhlB, which encode the fully functional enzyme . The amino acid sequence of RhlA has revealed a putative signal peptide at the N terminus, and there are at least two putative membrane-spanning domains in the RhlB protein (Ochsner et al. 1994). This suggests that RhlA is in the periplasm and RhlB is in the cytoplasmic membrane. In studies involving heterologous host expression (*E. coli* and other P*seudomonads*) of amnosyltransferase , enzyme activity was observed when just the rhlB gene was induced, indicating that the RhlB protein is the functional enzyme. However, levels of rhamnolipids in the supernatant of induced Pseudomonas cultures was significantly higher when both rhlA and rhlB genes were expressed, indicating the involvement of RhlA protein in the activity of RhlB. that RhlA may be involved in the synthesis or transport of precursor substrates for rhamnosyltransferase or in stabilization of RhlB in the cytoplasmic membrane . Biosurfactant production in P. aeruginosa is tightly regulated and under the control of a QS system (Sullivan 1998). The LasR (LuxR-type protein)/LasI oxo-C12-HSL) synthase pair regulate the expression of a large regulon that includes virulence factors such as the elastase gene, lasB, and a second signalling pair RhlR ,RhlI (C4-HSL synthase) . In the case of rhamnolipid synthesis, the transcription on rhlAB is under the control of RhlR and the signal molecule C4-HSL. rhlR and rhlI are located immediately downstream of the rhlAB. The provision of RhlR and C4-HSL is not sufficient for RhlAB expression, because further levels of regulation exist to silence this part of the quorum response in *P. aeruginosa* . Experiments in *P. aeruginosa* and *E. coli* have shown that transcription from the rhlAB promoter does not occur in logarithmic growth, even when the presence of RhlR and C4-HSL is verified. Additional levels of negative regulation are a common feature of genes encoding elements of the quorum response in P. aeruginosa DksA. In the case of RhlAB expression, there is a requirement for the stationary phase sigma factor, RpoS, as well as RhlR and C4-HSL. In addition ,RhlR also binds to the rhlAB promoter in the

absence of C4-HSL as a repressor *P. aeruginosa* is a bacterium that can adapt to relatively diverse environments and situations. The ability to produce rhamnolipid has been demonstrated to be an advantage to *P. aeruginosa* cells colonising various environments by promoting surface motility maintaining biofilm channels and rapidly killing neutrophils attracted to sites of *P. aeruginosa* infection.

QS and Virulence

The roles of population size, evasion of host defences, and QS are entwined in the control of pathogenicity of at least two important pathogens, *S. aureus* and *P. aeruginosa .*

Both organisms are common in our environment and are responsible for a wide range of infections. Often these infections are hospital acquired and are difficult to treat because of antibiotic resistance (Bonomo and Szabo 2006; Schito 2006). The pathogenesis of both species relies on the coordinated expression of multiple virulence factors, a process in which QS, via acyl-HSLs for *P. aeruginosa* and via modified peptides for S. aureus, has a central role. Allied with this is the capacity of both organisms to form infection-related biofilms.

A biofilm is a persistent mode of growth at a surface within a polymeric matrix exhibiting a resistant physiology. The bacterial cells within a biofilm are at high cell densities, and cell-to-cell signalling has been shown to play a central regulatory role in the development of a mature, resistant biofilm.

QS Is Essential for the Full Virulence of *P. aeruginosa*

P. aeruginosa uses a multilayered hierarchical QS cascade that links Las signaling LasR/LasI/3-oxo-C12-HSL), Rhl-signalling (RhlR/RhlI/C4-HSL), 4-quinolone signaling PQS, and genetically unlinked LuxR-type regulators, QscR and VqsR, to integrate the regulation of virulence determinants and the development of persistent biofilms with survival under environmental stress. The quorum response of P. aeruginosa is extensive and provides for the coordinated activation of major virulence determinants .

The quorum response can be subdivided into genes (1) that are induced only by 3-oxo-C12-HSL, (2) that are induced only by C4-HSL, (3) that are induced either by C4-HSL or 3-oxo-C12-HSL, and (4) that are only induced by C4-HSL and 3-oxo - C12-HSL , and the quorum response.

More importantly, it has been possible to show that acyl-HSL signaling is essential for the development of full virulence by *P. aeruginosa* during an infection .The effect of specific mutations in rhlI, lasR, and lasI has been investigated in murine models of acute pulmonary infections and burn wound infections.

In the burn wound model ,lasR, lasI, and rhlI mutants are significantly less virulent than the parent P. aeruginosa strain, PAO1 . After 48 hours, the wild-type strain shows an average mortality of 94% compared with mutants of lasR 28% mortality lasI (47%), rhlI (47%), and lasI, rhlI double mutant (7%). The virulence of the mutants was restored by complementation with plasmids expressing LasI, RhlI, or LasI and RhlI .

The virulence of *P. aeruginosa* is linked to the production of exoproducts that degrade tissue and allow the spread of bacteria to deeper tissue. To assess the spread of P. aeruginosa within the burned skin, bacterial counts were made at the site of inoculation and at a site 15-mm distant. Single rhlI and lasI mutations had no significant effect on the spread of the bacteria, mutants with defects in lasR or both rhlI and lasI showed no spread to the distant site until after 16 hours from inoculation .

These data suggest that although there is some redundancy in the control of the important virulence factors via las and rhl signaling, QS is necessary for the optimal coordination of virulence factor expression for pathogenicity .

A similar situation is apparent in the pulmonary infection model. Of the mice inoculated with the parental strain, 55% developed confluent pneumonia throughout the lungs, with a mortality rate of 21% of the inoculated animals. In contrast ,only 10% of mice inoculated with a rhlI, lasI double mutant developed pneumonia ,and

this was much less severe than that seen with the parent strain. Full virulence could be restored to the double mutant by complementation of the rhlI, lasI mutations with plasmid-borne copies of rhlI and lasI. In agreement with a role for signalling in pulmonary infection, although a lasR mutant could colonise the murine lung, it was avirulent, being unable to achieve high cell densities, cause pneumonia, or penetrate into deeper tissues .

The various QS signals of *P. aeruginosa* coordinate the expression of many individual phenotypic traits to present a bacterial population best able to survive within the confines of an infection. The use of different signals and response regulators provides for flexibility in the timing of the deployment of individual gene groupings and the integration of this transcriptional activation with other signals from the bacterial environment. Moreover, the signals of *P. aeruginosa* provide more than QS capabilities influencing immune responses, vasodilatation, and other bacterial species. QS Is Essential for the Full Virulence of *S. aureus* S. aureus is an opportunistic pathogen deploying a range of adhesions, evasions, and aggressions .The collection of genes expressed during an infection that is required for the establishment and progression of disease have been termed the “virulon.” The controlled expression of the virulon during an infection is central to the development of disease. The regulation of expression relies on the response to changing conditions resulting from penetration into host tissues and the resultant changes that occur because of bacterial and immune activities. The study of staphylococcal virulence has helped develop the concept of “antipathogenic “drugs these compounds do not kill the bacteria, but simply inhibit the expression of destructive virulence factors Agr, QS, and RNAIII.

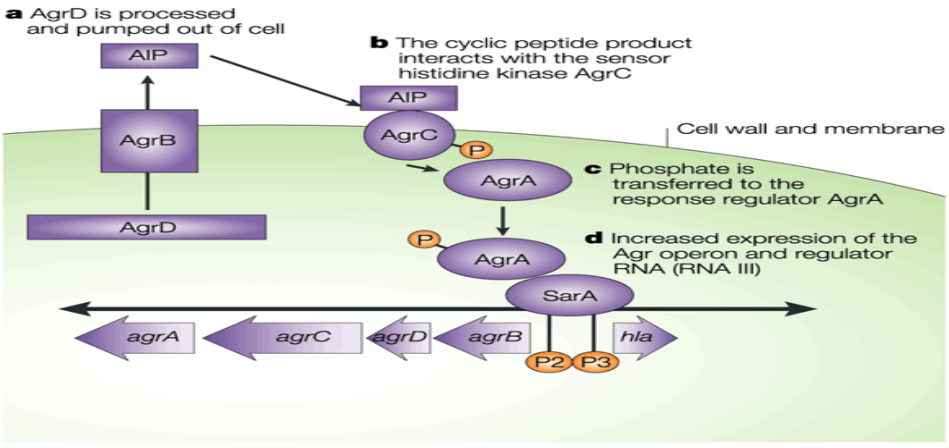
The virulon of *S. aureus* can be classified as surface factors (involved in adhesion and immune evasion, e.g., protein A) and secreted factors (toxins and enzymes involved in damaging the host, e.g., haemolysin, toxic shock syndrome toxin [TSST], and proteases. A pleiotropic transposon mutant that was downregulated for secreted

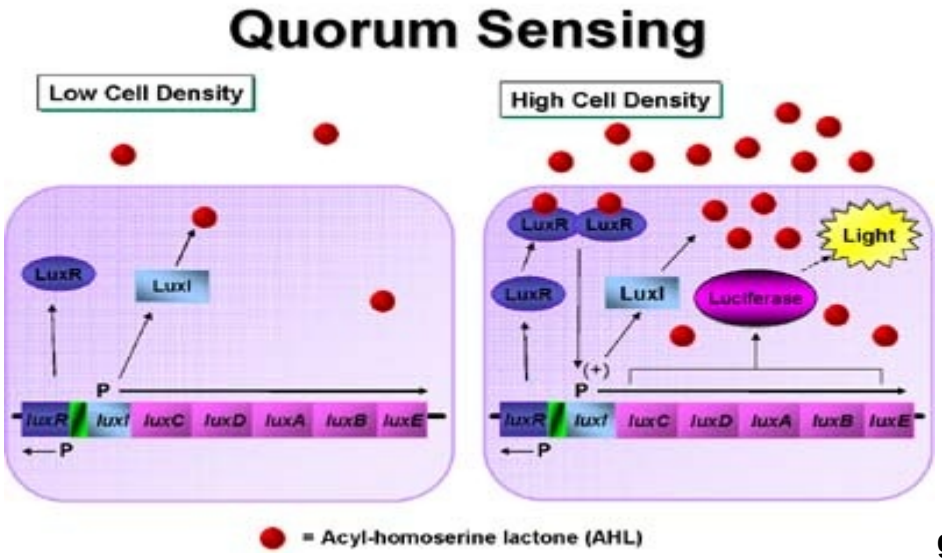
factors and upregulated for surface factors was first described in 1986 and has since been characterized in great detail. The mutation is in the accessory gene regulator region in agrA. The control of gene regulation through agr is in response to increasing bacterial cell density.

During the initial, low population-density stages of a staphylococcal infection the expression of surface proteins binding extracellular matrix molecules, e.g fibronectin, collagen, and fibrinogen, and to the Fc region of immunoglobulin, i.e Protein A, is favoured. This is thought to promote evasion of host defences and the successful colonization of host tissues. *S. aureus* challenges the host immune system by eliciting a regional inflammation and subsequent abscess formation Inside the effectively closed system of the abscess, bacterial population density increases and secreted enzymes and toxins are induced that efficiently destroy white blood cells and liberate nutrients from tissue .

The Agr locus consists of two divergent operons, P2 and P3 .The P2 operon comprises the agrBDCA signaling cassette. P3 encodes the RNAIII molecule that acts as an intracellular signal controlling the transcription of genes within the Agr regulon. AgrD encodes a small peptide that is cleaved and processed in a process that involves AgrB, and which results in the secretion of a thiolactone peptide or AIP.

Figure 33. Mechanisms of quorum sensing.





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Signal transduction in bacterial chemotaxis

Introduction

Motile bacteria respond to environmental cues to move to more favorable locations. The components of the chemotaxis signal transduction systems that mediate these responses are highly conserved among prokaryotes including both eubacterial and archael species. The best-studied system is that found in *E coli*.

Attractant and repellant chemicals are sensed through their interactions with transmembrane chemoreceptor proteins that are localized in multimeric assemblies at one or both cell poles together with a histidine protein kinase, CheA, an SH3-like adaptor protein, CheW, and a phosphoprotein phosphatase, CheZ. These multimeric protein assemblies act to control the level of phosphorylation of a response regulator, CheY, which dictates flagellar motion. Bacterial chemotaxis is one of the most- understood signal transduction systems, and many biochemical and structural details of this system have been elucidated. This is an exciting field of study because the depth of knowledge now allows the detailed molecular mechanisms of transmembrane signaling and signal processing to be investigated.

Microbiology began with the advent of light microscopy in the 17th century. living organisms because of their purposeful motions. By the end of the 19th century, the motor responses of bacteria had been thoroughly characterized by numerous investigators including the great German physiologist, Wilhelm Pfeffer. This research established that bacteria move in response to changes in temperature (thermotaxis), light (phototaxis), salinity (osmotaxis) and oxygen (aerotaxis), and to specific metabolites and other signaling molecules (chemotaxis). It was not until the end of the 20th century, however, that the molecular mechanisms that underlie bacterial sensory-motor regulation had been established.

In the 1960s, the mechanism of chemotaxis in *E. coli* established that *E. coli* chemotaxis responses to amino acids and sugars are mediated by receptors at the

cell surface that relay information via an intracellular signal transduction network to effect appropriate changes in motor behavior.

The components of the intracellular signal transduction machinery were defined through the isolation and mapping of hundreds of different *che* (chemotaxis) mutants. Molecular genetic approaches initiated by Silverman and Simon in the 1970s established a bridge from che genes to Che proteins.

By the 1980s, it was possible to reconstitute the entire *E. coli* chemotaxis signal transduction system in vitro from its purified component parts. Atomic resolution structures are now available for several receptor fragments and all six *Che* proteins of the *E. coli* chemotaxis system.

Recent research has largely focused on the way that these components are organized in the bacterial cell and how signals are transmitted across the membrane. It had generally been assumed that each membrane receptor interacted with a small complement of *Che* proteins to produce its own signal. The motor output was thought to represent a summation of the inputs from several thousand independent receptor–signaling units scattered over the surface of the cell.

Chemotaxis behavior

It has become customary to regard living systems as machines. The *E. coli* flagellar motor fits well with this type of analogy. It is a nanoscale device that operates at close to 100% efficiency. Embedded in the bacterial cell, each motor uses electrochemical energy to rotate a long helical flagella filament that propels the bacterium. A typical cell has a complement of half a dozen or more flagella anchored to independently rotating motors randomly distributed over the surface of the cell. Each motor alternates between clockwise or counterclockwise rotation with switching frequencies that exhibit the stochastic features of a two-state thermal equilibrium. Hydrodynamic drag causes counterclockwise rotating flagella to come together to form a bundle that acts cooperatively to push the cell body at speeds of approximately 20 microns per second. This behavior is termed smooth swimming or

running.

If one or more motors switch to rotate clockwise, the flagella become uncoordinated and the bacterium tumbles in place. In a uniform environment, cells move in a random walk: running

for about a second, then tumbling for about a tenth of a second, then running in a random new direction. If a cell detects increasing concentrations of attractants or decreasing concentrations of repellents, its tendency to tumble is reduced, biasing its overall motion towards attractants and away from repellents. In a sense, chemotaxis towards attractants and away from repellents is determined by the cumulative effects of the second-to-second decisions of each individual to continue swimming or to tumble and change direction.

The large polar assemblies of receptors and Che proteins function to control the probability that a cell will tumble and change direction. There is no simple relationship between the enormous quantities of sensory information received by these structures and the signals that they generate to control motility.

Research has focused on properties of bacterial behavioral responses that are widely shared by other organisms.

To a first approximation, they respond only to changes in the concentration of an attractant or repellent chemical rather than to absolute levels. After a short period (seconds to minutes) of continuous exposure, they behave as if no stimulus were present. Bacteria have memory and can learn. The response of each bacterium to a given stimulus is entirely dependent on the history of that particular cell. Bacteria are individuals, each with a unique character. Some are more generally tumbly than others. Some are more smooth swimming. Responses to stimuli vary enormously from cell to cell.

The molecular mechanisms that underlie these behaviors are now beginning to be understood in some detail.

Overview of *E. coli* chemotaxis signaling

Virtually all, motile prokaryotes use a two-component signal transduction system with conserved components to regulate motor activity. In general, a two-component system includes a histidine protein kinase (HPK) that catalyzes the transfer of phosphoryl groups from ATP to one of its own histidine residues and a response regulator that catalyzes transfer of phosphoryl groups from the HPK-histidine to an aspartate residue on the response regulator. In the chemotaxis system, the histidine kinase, CheA, associates with a distinct class of transmembrane receptor proteins, termed chemoreceptors, which interact with chemicals in the surrounding environment. Together chemoreceptors, CheA, and a third protein, CheW, form large receptor–signaling complexes that integrate sensory information to control CheA kinase activity. By regulating CheA autophosphorylation, receptor–signaling complexes control the phosphorylation of the chemotaxis response regulator, CheY. CheY reversibly binds CheA, dissociates from CheA upon phosphorylation, and rapidly diffuses to flagellar motors. At the motor, phospho-CheY acts as an allosteric regulator to promote clockwise rotation and tumbling.

The primary output of the E. coli chemosensory apparatus is phospho-CheY. Chemotaxis results from the modulation of the concentration of phospho-CheY present in the bacterial cells that are swimming in gradients of attractant and repellant chemicals. Attractant stimuli suppress tumbles by interacting with chemoreceptors to inhibit CheA kinase activity and thereby decrease phospho-CheY. The concentration of phospho-CheY is also affected by three soluble enzymes that are peripheral components of the sensory system: CheZ, CheR and CheB. CheZ is a protein phosphatase that associates with the receptor–signalingcomplexwhereitacts toenhancetherateof phospho-CheY dephosphorylation.

CheR and CheB are enzymes that methylate and demethylate the cytoplasmic portion of each chemoreceptor. CheR is an S-adenosylmethionine-dependent methyltransferase that methylates specific glutamate side chains, converting these carboxylate anions into uncharged methyl esters. CheB is an esterase that

hydrolyzes the methyl esters formed by CheR to restore negatively charged glutamates. CheB also deamidates specific glutamine groups to produce glutamates that are then subject to esterification by CheR. The activities of CheR and CheB are regulated by the activity of the receptor– signaling complexes to generate changes in the chemoreceptor methylation and amidation levels that play a critical role in adaptation. The CheR and CheB modifications also provide a memory mechanism that alters behavioral responses to subsequent stimuli. The stochastic nature of these modifying activities also ensures that no two cells will have precisely the same complement of receptor sensitivities.

The six essential Che proteins, CheA, CheW, CheY, CheZ, CheR and CheB together with five chemoreceptors, Tsr, Tar, Tap, Trg and Aer, collectively constitute the *E. coli* chemotaxis system . Differences in the number of copies of these genes and fusions and deletions of Che proteins represent numerous variations on this theme in different bacterial and archael species.(10,29) Nevertheless, in virtually all motile prokaryotes, receptor–signaling complexes composed of homologous chemoreceptor proteins, CheWs and CheAs act together with homologous CheYs to control sensory-motor activities. In order to understand the function and regulation of these conserved receptor–signaling systems,first weprovide a discussion of the individual structures and functions of the chemoreceptors, CheA, CheW and CheY found in *E. coli.*

E. coli sense attractant and repellant stimuli via five chemoreceptor proteins: Tsr, Tar, Tap, Trg and Aer. These transmembrane proteins are composed of highly variable periplasmic sensing domains that interact with stimulatory ligands, and a conserved cytoplasmic domain that provides a scaffold for CheW and CheA binding. The sensing domain forms an up-down-up-down four helix bundle. The cytoplasmic domain is divided into three subdomains: the HAMP domain, the methylated helices, and the signaling domain. Molecular models of the dimeric sensing domain of Tar and a dimer of the truncated cytoplasmic domain of Tsr (residues 290–514) are

shown to the right. The sensing domain of the aspartate receptor, Tar, has been expressed from the corresponding fragment of the tar gene and purified as a soluble homodimer.(34,35) The X-ray crystal structure indicates that each monomer is composed of an up-down-up-down four-helix bundle. In the cell, the first and last helices of this four-helix bundle extend across the membrane into the cytoplasm. The C-terminal end of the last helix (the second transmembrane helix) is linked to the signaling domain in the cytoplasm. The Tar sensing domain homodimer has two symmetrical, non-overlapping aspartate binding sites at the dimer interface. Binding of aspartate to either symmetric site causes a conformational change that precludes binding at the second site. Further ligand-induced conformational changes such as a downward piston-like movement of the second transmembrane helix with respect to the first transmembrane helix and a rotation of dimer subunits with respect to one another may be the source of signaling across the cell membrane for inhibition of CheA kinase activity in the cytoplasm.

Although the chemoreceptor sensory domains are variable and specialized for ligand binding, they are all linked to a conserved cytoplasmic domain that extends away from the membrane and then bends back on itself via a hairpin turn. The degree of sequence identity is at a maximum in the hairpin turn region and decreases away from the center giving variable sequences. The cytoplasmic domain structure can be divided into four subdomains beginning at the N terminus the Histidine kinases, adenylyl cyclases, methyl-binding proteins and phosphatases' domain (HAMP), methylated helix 1 (MH1), the highly conserved domain or signaling domain, and methylated helix 2 (MH2). The HAMP domain sequences have very little primary sequence identity but biochemical investigations of Tar support sequence-based structure predictions that the HAMP domain consists of two amphipathic helices connected bya non-helical or globular structure. The HAMP domain is followed by a long, antiparallel alpha-helical coiled coil with MH1 and MH2 juxtaposed above the conserved signaling domain.Together the methylated helices (MH1 and MH2)

contain four or more glutamyl residues that are substrates for CheR and CheB modification.These residues are spaced in heptad repeats along one face of each helix. CheA and CheW interact with chemoreceptors in the region of the highly conserved signaling domain. The structure of a soluble fragment of Tsr, encompassing the methylated helices and the highly conserved domain, has been solved by X-ray crystallography. The solved structure confirms experiments and predictions that the long protein fiber is an antiparallel coiled coil that forms a four-helix bundle when dimerized. This crystallographic data, in addition to biochemical crosslinking data, show that multiple four-helix bundles come together into trimers of dimers contacting one another within the signaling domain.

CheA

CheA is the largest and most-complex component of the chemotaxis system. It is divided into five structurally and functionally distinct domains: the histidine phosphotransfer domain (P1), the response regulator binding domain (P2), the dimerization domain (P3), the histidine protein kinase catalytic domain (P4), and the regulatory domain (P5). These five domains are numbered in order from the amino to the carboxy terminus.

The P1 domain belongs to the histidine phosphotransfer family of proteins that function as intermediates in the transfer of phosphoryl groups between ATP and the phospho-accepting aspartate side chains of response regulators. Other HPt domains of known structure include Ypd1 from *Saccaromyces cerevisiae* and ArcB from *E. coli*. All of these HPt proteins consist of an up-down-up-down four-helix bundle. Despite their structural and functional similarities, the sequences of HPt proteins are poorly conserved and difficult to detect by sequence alignment. The phosphorylated histidine, however, is invariably located in a solvent exposed position on the second helix of the four-helix bundle, and conserved glutamate and lysine residues surround the active site. In CheA, phosphorylation occurs on the N3 nitrogen of the imidazole side chain of His48. P1 can be expressed from the corresponding fragment of the

cheA gene and purified to yield a soluble monomeric protein. The isolated P1 domain can be phosphorylated by the HPK catalytic core (i.e. the P3 and P4 domains), and the phosphorylated product, phosphoP1, retains its CheY-phosphotransfer activity. Even though the active site glutamate and lysine residues are essential for ATP-dependent phosphorylation of P1, they are not required for phosphotransfer between P1 and CheY.

The response regulator binding domain, P2, is flanked by two flexible linker sequences connecting it to P1 and P3.Like P1, P2 can be produced as an independent monomeric protein. The structure of P2 shows four antiparallel beta-sheets and two oppositely oriented alpha-helices.

When P2 is in complex with CheY, the CheY active site undergoes a conformational change that increases the accessibility of the phospho-acceptor aspartate, Asp57. More importantly, P2 binds CheY in close proximity to the phosphoP1 domain and increases its effective concentration. Sequences that are homologous to P3 and P4 have been identified in over a thousand different signal transduction proteins. These two domains constitute the histidine protein kinase (HPK) catalytic core. Expression of P3–P4 from the corresponding portion of the cheA gene produces a protein that phosphorylates P1 at rates comparable to those obtained. A molecular model of the histidine protein kinase—CheA. The histidine phosphotransfer domain (P1) and the response regulator CheY/CheB-binding domain (P2) are depicted as monomers connected to one another and the remainder of CheA via flexible linkers. The dimerization domain (P3), ATP-binding phosphotransfer domain (P4), and the receptor-binding domain (P5) are all depicted within a CheA dimer. Models were generated using coordinates taken from Mourey et al, McEvoy et al, and Bilwes et al using Swiss PDB Viewer. The P3 domain is a long antiparallel coiled coil that forms a symmetric up-down-up-down four-helix bundle in the CheA dimer; hence, the P3 four-helix bundle has essentially the same fold as the chemoreceptor cytoplasmic domain dimer . In solution, CheA homodimers and monomers are in equilibrium (KD

0.2 mM). Whereas the monomers are inactive, dimers exhibit a basal rate of ATP-dependent histidine phosphorylation. In most HPKs, a dimerization domain corresponding to P3 contains the site of histidine phosphorylation within a conserved sequence that has been termed the H-box. The X-ray crystal structure of the CheA-P3 domain is very similar to the nuclear magnetic resonance (NMR) solution structure of the phosphoaccepting dimerization domain of the archetypal HPK, EnvZ. Although some of the conserved H-box residues in EnvZ are retained in CheA, the CheA dimerization domain is not phosphorylated. The residue corresponding to the phospho-accepting histidine in the EnvZ H-box is a glycine in CheA. The only site of phosphorylation in CheA is the His48 side chain in P1. Nevertheless, the CheA dimerization domain appears to play almost as important a role in CheA histidine kinase activity as it does in HPKs like EnvZ. Although the catalytic ATP-binding P4 domain appears to be an independent unit that does not participate in dimeric interactions, the CheA-catalytic core must be dimeric to phosphorylate P1. It seems likely that, in CheA, the dimeric P3 domain fuctions to bind P1 and position it for phosphorylation by ATP bound to P4. The P1 domain of a CheA subunit that has a defective kinase catalytic domain is readily phosphorylated in trans by a CheA subunit that has a defective P1 domain and an active kinase catalytic domain. Trans phosphorylation, which has also been shown for a number of HPKs, does not, however, explain the need for dimerization.

Receptor–signaling complexes

Determination of the structures of the chemoreceptors and all the Che proteins has provided a foundation for understanding the assembly of receptor–signaling complexes. It was initially assumed that CheW monomers bound to receptor–signaling dimers, and then CheA dimers bound to the receptor-associated CheWs to form 2:2:2 complexes. Each 2:2:2 complex was thought to work independently to modulate the rate of CheA autophosphorylation. The outputs from the thousands of these complexes in a single cell were thought to be summarily integrated through

their effects on a common pool of phospho-CheY in the cytoplasm. More recent findings indicate that this model is incorrect. Now it is clear that thousands of chemoreceptor proteins in a single *E. coli* come together to form one or two large interconnected arrays at one or both poles of the cell. In *E. coli* the five chemoreceptors, of varying sensory specificities, interact cooperatively to regulate CheA kinase activity and are expressed together with all of the che genes at roughly fixed ratios of one to another.

Signaling across the membrane

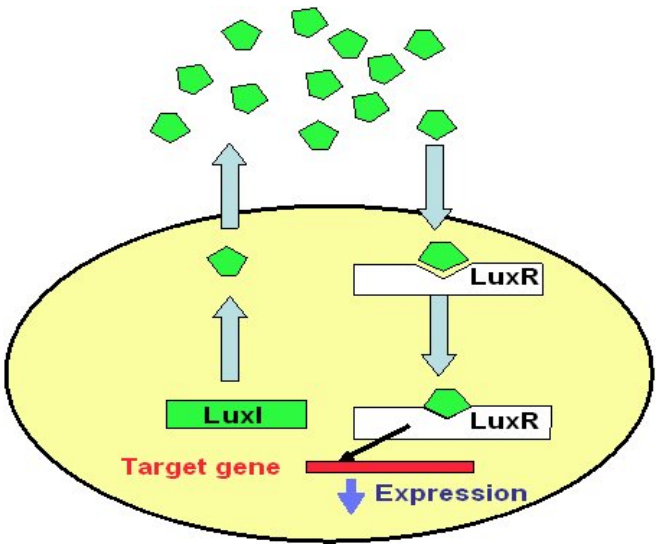
There have been many theories as to how stimuli from the extracellular environment are perceived and how this information is used to effect excitatory and adaptive responses.

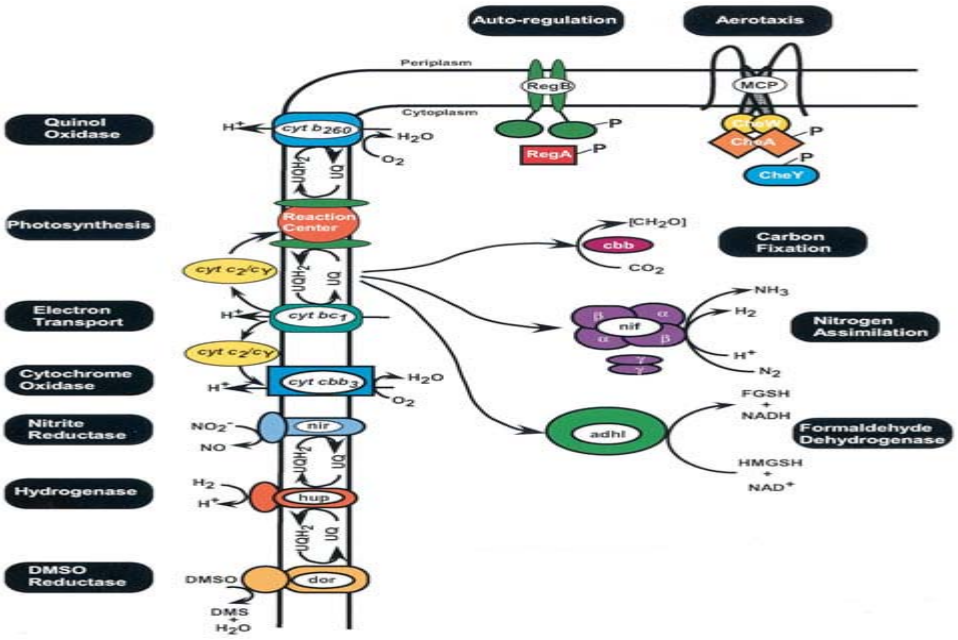
Hypotheses about the mechanism of transmembrane signaling in the *E. coli* chemotaxis system were initially derived from detailed analyses of the conformational changes that occur when aspartate binds to Tar. It is apparent that aspartate binding causes a significant movement of one subunit with respect to the other within a receptor dimer. In addition to these small inter-subunit displacements, there are substantial changes over the entire solvent-exposed surface of the dimer as well as changes in the orientation of the dimer with respect to the plane of the membrane. All of these perturbations would be expected to promote disorder within the sensory array favoring expansion of the array of signaling domains and their associated CheAs and CheWs-on the other side of the membrane.

Aspartate binds along one side at the juncture between dimers. Aspartate binding at either of the two equivalent sites obstructs the other site. The KD for aspartate binding to receptor domain dimers in solution is approximately 1 mM. Assuming the rate of binding is diffusion limited, the half-life of an individual aspartate-bound receptor is about a millisecond.

Once an aspartate molecule enters a sensory domain array, it will tend to bind numerous times to numerous different receptors. EM images of receptors in membranes indicate tightly packed highly ordered structures. Each aspartate-binding event would tend to disrupt such organized arrangements. Chemotaxis signal transduction can be approximated by two-state formalism. Thus the structure of the sensing domain array may be considered in terms of an equilibrium between two states—an ordered, tense or T state and a disordered, relaxed or R state, with aspartate binding with slightly higher affinity to sensory domains in the R state. The structure of the receptor–signaling complex in the cytoplasm may also be considered in terms of an equilibrium between two states—a highlycondensed Tstate where CheA is fully active, and a relatively diffuse R state where CheA is inactive. As a first approximation, one can assume a one-to one correspondence between the R !T equilibrium of the input sensing array and the R !T equilibrium of the signaling-complex output. Using such a formulation, one can obtain a good fit of receptor-mediated stimulus–response coupling.

Figure 34. Mechanism of signal transduction in bacteria.




Auto-regulation
Aerotaxis
Periplasm
Cytoplasm
RegB
MCP
RegA
CheA
CheY
Quinol Oxidase
Photosynthesis
Electron Transport
Cytochrome Oxidase
Nitrite Reductase
Hydrogenase
DMSO Reductase
cyt b560
Reaction Center
cyt c2/cy
cyt bc1
cyt cbb3
nir
hup
dor
UQ
Carbon Fixation
cbb
Nitrogen Assimilation
nif
Formaldehyde Dehydrogenase
adhI
FGSH
NADH
HMGSH
NAD+
DMSO
DMS

Mitochondrial DNA

Introduction

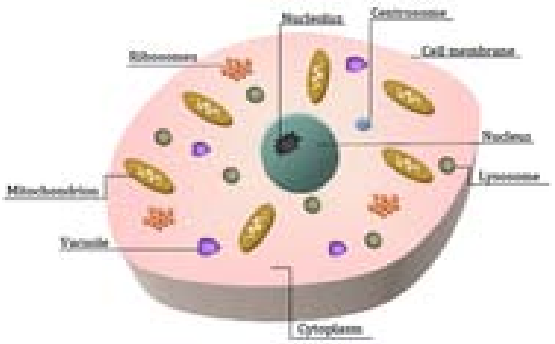
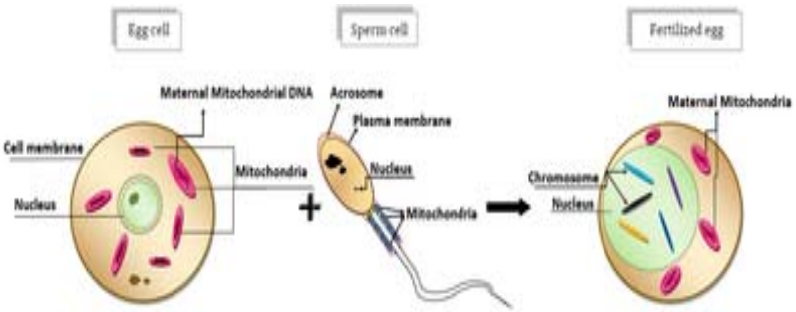


Illustration of a eukaryotic cell with contents labeled Mitochondrial DNA (mtDNA), is a small circular DNA molecule located in mitochondria which are cell's organelles residing in the cytoplasm. It is approximately 16,569bp (base pairs) long and consists of 37 genes and a control region which is also called a 'non-coding' region since it does not code for any gene products.

Maternal inheritance

Unlike nuclear DNA, mtDNA is inherited strictly from mother to child. Any sperm mitochondria that might enter the fertilised egg are destroyed by embryo's cellular machinery due to a tag that is added onto them during spermatogenesis which marks down the sperm mitochondria for degradation. Thus mtDNA is not unique for each individual since all the maternal relatives have identical mtDNA sequence.

Figure 35. Inhereitance of mitochondrial DNA.



However, mitochondrial DNA is not the same between all populations and this is due to lack of repair mechanisms and proofreading capabilities which makes it susceptible to base substitutions, leading to high mutation rates. The mutation rates of mitochondrial DNA are 10 times higher than in nuclear DNA.

This provides mtDNA with variability useful in human identity testing and in investigating the evolutionary relationships among individuals and species, by interrogating its short variable sections. These variable sections are located in the control or 'non-coding' region of mtDNA; given that it does not code for any gene products, the limits for nucleotides mutation are fewer and the rate of polymorphisms is high between each person.

Human mtDNA

Human mtDNA was first sequenced in Sanger's laboratory in Cambridge in 1981. This first sequence was called 'Anderson' or Cambridge reference sequence (CRS) and for many years the new sequences were compared with it. In 1999 it was revised by Andrews who confirmed almost all of the original identified nucleotides. The sequences across HV1 and HV2 that are most commonly used in forensic applications were found to be identical.

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