Investigation of bacteria by genetic methods

Delivered by;>Annu R .patel
M.Sc(Microbiology)sem-2
Roll no.-9
Guided & checked by; Dr. Samir .c. Parikh

P. G. center of microbiology
Smt. S. M. Panchal science College,Talod

Genetic Methods;

- Microbial Physiology,
- Bacterial virulence,
- Specific mutagenesis,
- Taxonomy,
- Evolution & Epidemiology.

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Introduction

- ✓ Genetics has been central to the development of our understanding of bacterial metabolism and physiology and some examples will be reviewed in this seminar.
- ✓ Although genomics receives most of the publicity, the techniques in this seminar are important for understanding the function of the genes concerned in relation to the overall behavior of the cell.

Microbial physiology

- Bacterial celled have complex structures, such as flagella, ribosome's and bacterial cell envelopes, as well as sophisticated systems such as the control of replication and cell division or the control of lysogeny in temperate bacteriophages.
- Genetics therefore plays a central role in the investigation of such systems.

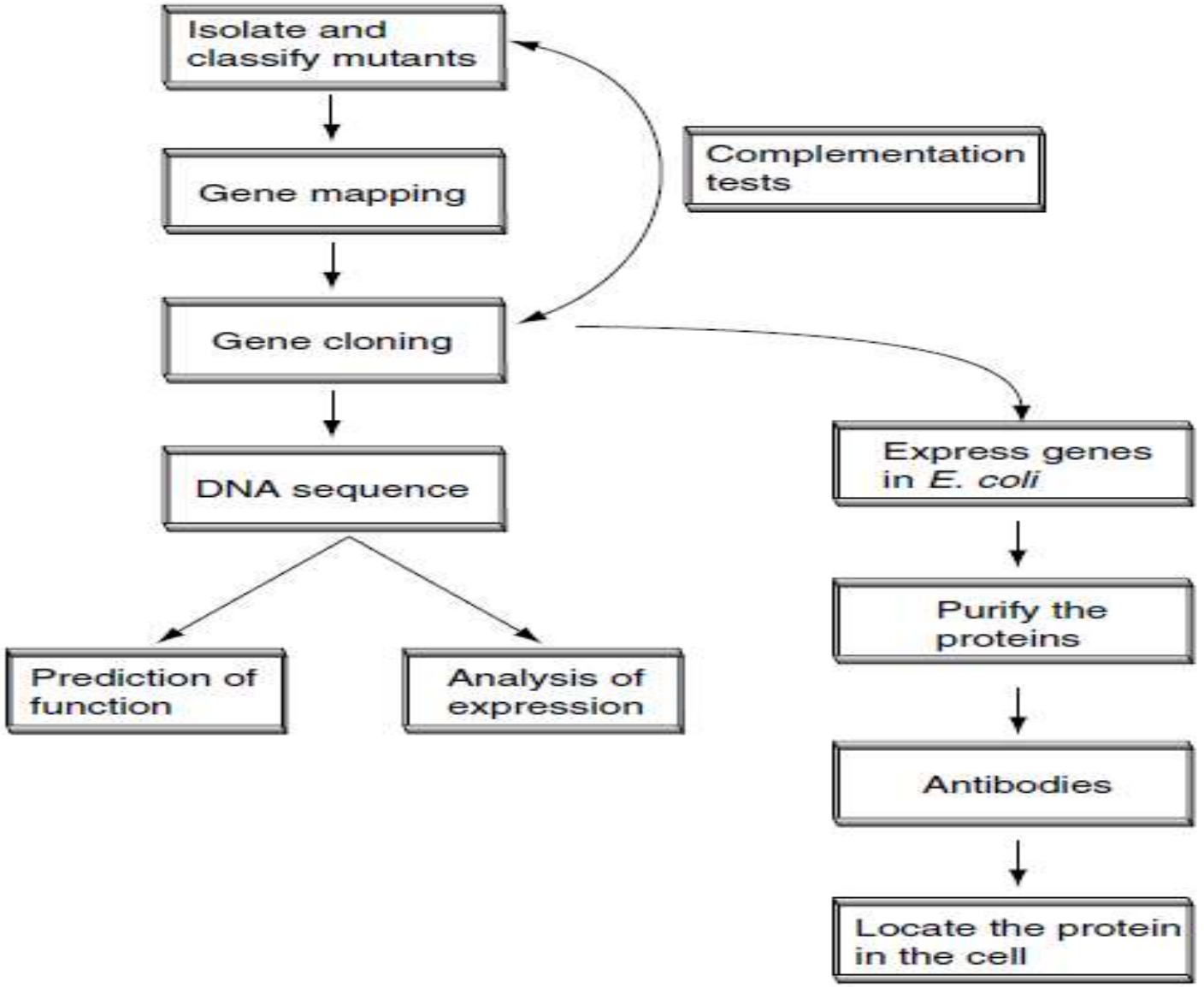

Figure 9.3 Techniques for genetic investigation of phenotypic characteristics. The flowchart illustrates some of the routes that start with specific mutants. There are many other methods of cloning and identifying genes

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- Some of the ways of analysing the genetic basis of physiological characteristics are summarized in Figure 1 The starting point in this case is the isolation of a series of mutants that are altered in a specific characteristic (such as sporulation for example). These mutants can be classified according to the precise nature of their phenotype, as well as by complementation analysis.
- This information can be used to identify the genes in a library. Probes can also be made, using the sequence data, to analyse the expression of the gene under different conditions, which may yield information as to its role.

1) Reporter genes

- A gene whose phenotypic expretion is easy to monitor used to study promoter activity in different tissue or devlopmental satage recombinant DAN contrast are made in which the repoter gene is atteched to a promoter region of particular intrest and the contrast transfer into a cell or orgenism
- A convenient and widely used method of reporter genes. This involves attaching the regulatory region of the gene concerned to another gene that is more easily detected so that the regulation by proxy can be followed by observing the expression of the reporter (Figure 9.4).

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- For example, if a *b-galactosidase reporter and a medium containing* the chromogenic substrate X-gal is used, the colonies will only turn blue when the promoter in question becomes activated and the reporter gene starts to be expressed.
- One use of reporter genes is to identify unknown genes whose expression is activated in response to a given stimulus. In this case, random fragments of DNA, some of which will contain promoter regions, are fused to the reporter gene to generate a library of promoter fusions.

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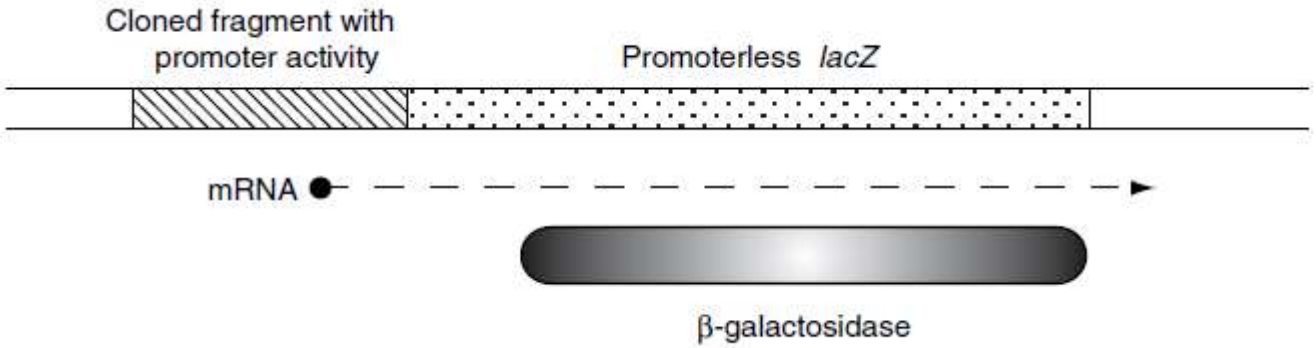
- The library is then plated onto a medium containing X-gal (assuming that *b-galactosidase is the reporter). The colonies of* interest are those which are white initially but turn blue when the conditions are changed – for example if the plate is transferred to an anaerobic incubator.
- This indicates that the promoter is responsive to the new environment which in this case is growth under anaerobic conditions. From this we can infer that the gene which is normally expressed from that promoter is one that is needed for



✓ Figure 9.4 Use of reporter genes. The diagram shows a transcriptional fusion, in which a promoterless lacZ gene is fused to a promoter, thus enabling the activity of that promoter to be characterized. For some purposes, translational fusions are used, where the promoter fragment also provides translational signals and the 50 end of a protein coding sequence, leading to a fusion protein containing the reporter anaerobic growth in the original host.

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- While this approach has been largely replaced by the advent of microarrays which enable global gene expression to be analysed more easily , it has been widely used to identify genes which are expressed in response to heat, starvation, osmotic shock and during sporulation for example.
- Apart from *b-galactosidase, two other reporter genes are worth a specific* mention. The expression of luciferase results in the production of blue-green light and this allows the expression of a gene to be monitored simply by measuring light production.

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- Another reporter gene that has additional utility is Green Fluorescent Protein (GFP), a protein (originating from the jellyfish Aequorea victoria) that is intrinsically fluorescent and emits a green light when exposed to ultraviolet irradiation. This has the advantage of being readily detected in situ, without the need for an enzymic substrate.
- In addition to its use as a reporter of gene expression, it can also be expressed as a translational fusion, so that the target protein is labelled with GFP. This enables the location of the target protein within the cell to be determined.

2) Lysogeny

- The biologicl process in which a becterium is infected by bacteriophage but integrats its DNA into that of the host such that the host is not destroy.
- One example lies in the control of lysogeny of bacteriophage lambda (*l).* Lambda is a temperate bacteriophage, which means that when it infects a sensitive strain of E. coli it can establish a more or less stable relationship with the host cell (lysogeny) and in this state the *l DNA is inherited by the daughter* cells at cell division.
- Its continued presence is indicated by the occasional breakdown of the lysogenic state which results in the production of bacteriophage particles.

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- Lysogens are resistant to infection by other lambda phage particles (they show superinfection immunity), so the plaques produced by infection of E. coli with *I are* normally turbid, rather than the clear plaques produced by a virulent phage (such as T4). Within the plaque, lysogenic bacteria will continue to grow, which causes the turbidity of the plaque. However, if enough plaques are examined, occasionally one will be found that is clear rather than turbid.

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- This is due to a mutant bacteriophage that is unable to establish lysogeny. These are known as clear plaque mutants, designated c (the designation of bacteriophage genes does not follow the normal three letter system that applies to bacterial genes). Complementation analysis shows that there are three genes involved, cI, cII and cIII. These mutants behave rather differently, in that cI mutants always kill the infected cell, while cII and cIII mutants can (albeit very rarely) give rise to stable lysogens.

3) Cell division

- The process of cell division is so central to bacterial multiplication that mutants would be expected to be non-viable. Surprisingly however, some mutants are still able to grow. Amongst these are the min mutants of E. coli, so-called because at cell division some of the daughter cells are small minicells that do not contain any chromosomal DNA (Figure).
- These minicells are of course non-viable, but the mutant is still able to multiply as some cell divisions occur as normal. These mutants have been invaluable in attempting to answer a key question relating to cell division: Why does cell division normally occur only at the central point of the cell and not elsewhere?
- The full answer is rather complex, but essentially the role of the products of the min genes is to inhibit cell division at sites other than the midpoint of the cell.

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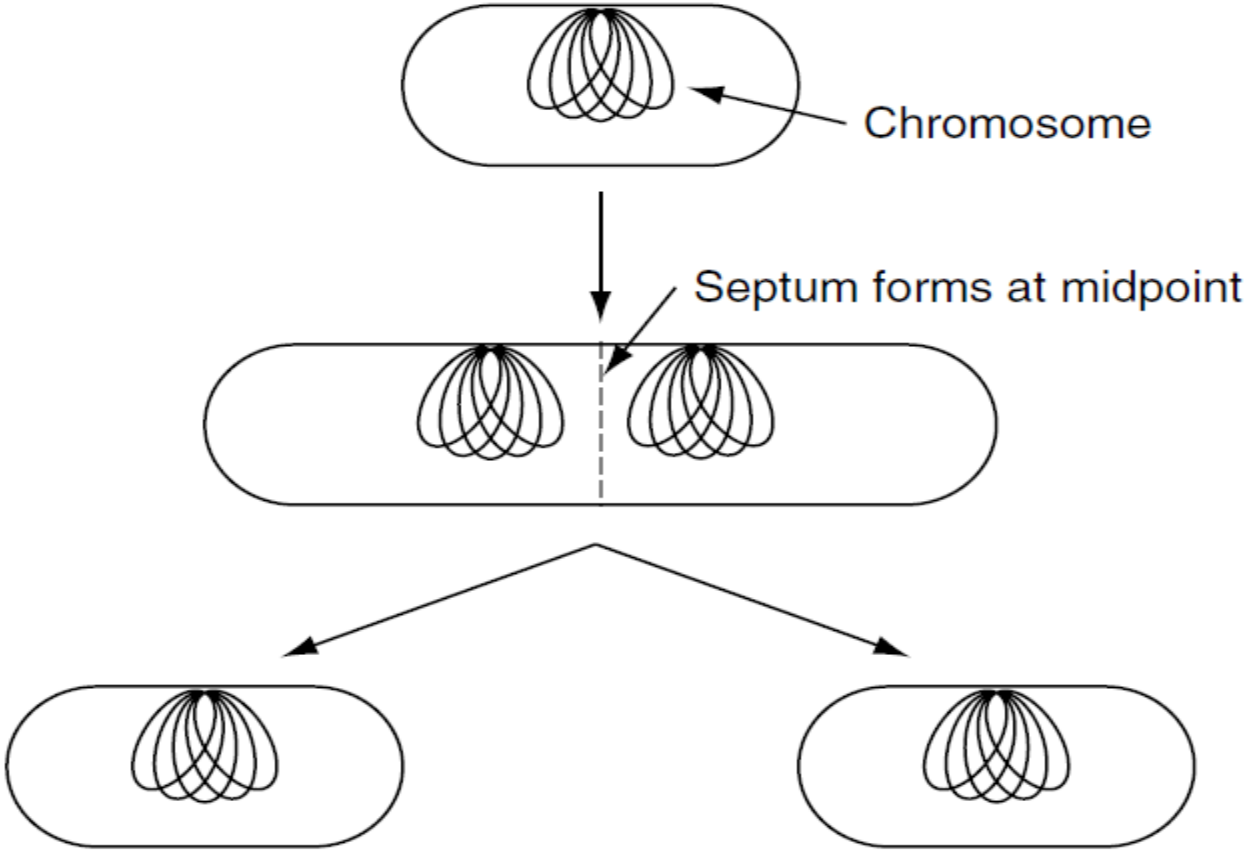
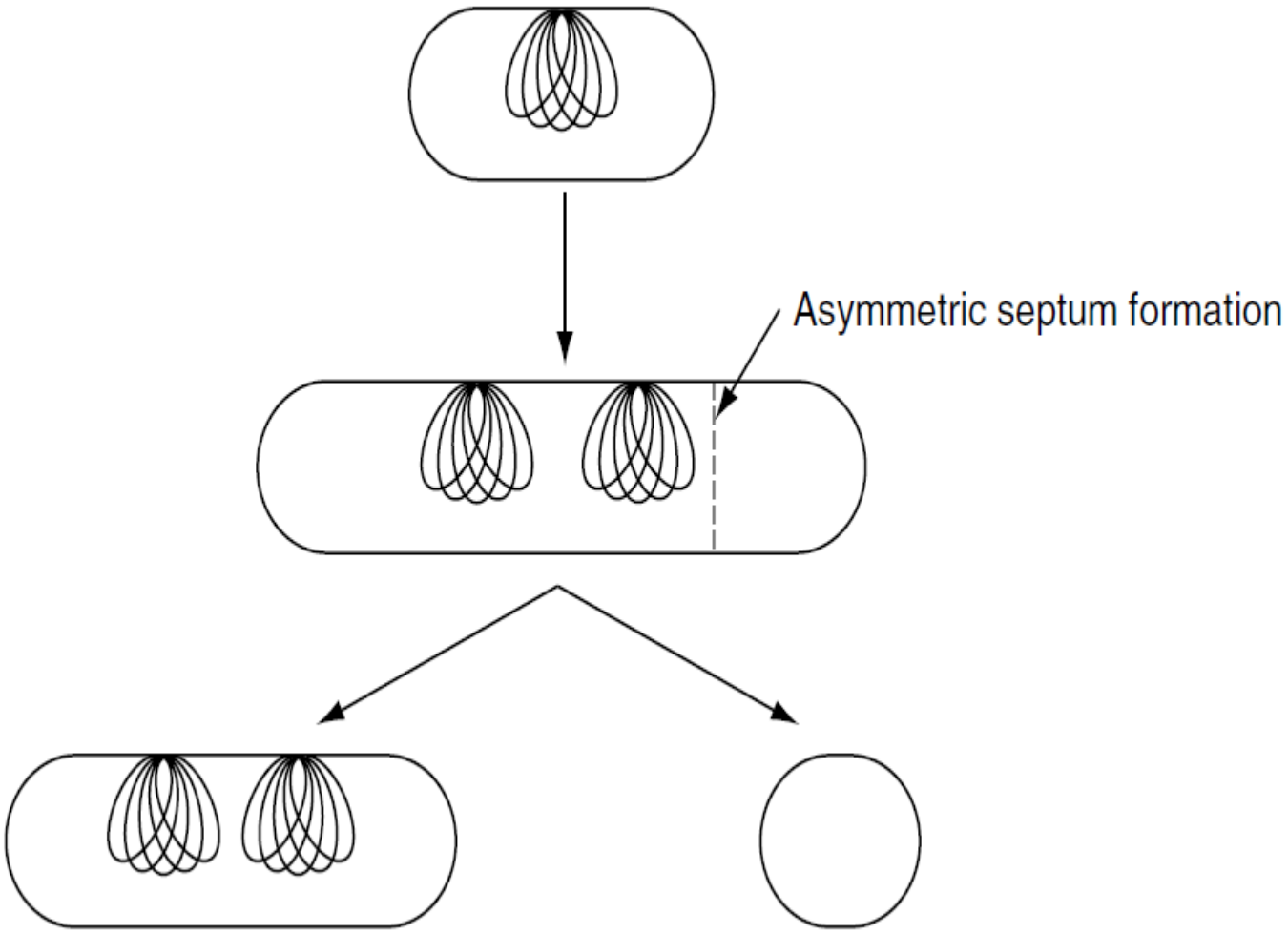
- Of course many mutations affecting cell division are lethal, therefore conditional mutants are used to study these genes – especially temperature-sensitive mutants. One of the most likely consequences of a failure of cell division in a rod shaped bacterium such as E. coli, is the formation of long filaments.
- Mutants that form filaments when grown at a higher temperature are known as fts mutants. Genetic analysis showed that mutation in any of a number of genes could give rise to this phenotype. One of the most important of these is the ftsZ gene.
- The techniques summarized earlier have established that the FtsZ protein initiates the formation of the septum that will divide the two cells by polymerizing into a ring-like structure at the site of cell division.

(a) Normal cell division



Cell division in *E. coli*: minicell mutants. (a) Cell division normally occurs at the midpoint of the cell.

(b) Cell division in a *min* mutant



(b) With a *min* mutant, some cell divisions occur asymmetrically producing a small (non-replicating) cell that contains no chromosomal DNA

4) Motility and chemotaxis

- In the absence of chemical or other stimuli, many bacteria including E. coli, swim about in an apparently random manner which is composed of periods of smooth motion in one direction (the run) interspersed by abrupt tumbling.
- This is related to the structure and control of the flagella which impart motion by rotating. When this rotation is counterclockwise, the filaments coalesce into a bundle which drives the cell evenly in a particular direction.
- Tumbling results from a reversal in the direction of rotation of the flagella. As soon as they start to rotate in a clockwise direction, the bundles of flagella fly apart, the cell tumbles briefly and then the flagella resume their normal counterclockwise rotation.

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- The cell therefore starts to swim smoothly again, but now in a different direction.
- The link with chemotaxis (the ability to swim towards, or away from, specific chemical stimuli) is in the control of the frequency of tumbling. When the cell is moving towards an attractant, the length of the run is increased (or the frequency of tumbling is decreased, which is the same thing).
- So those bacteria that happen to be swimming in the right direction will swim further before changing direction; those that are going the wrong way will change direction sooner.

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- Our understanding of this complex and fascinating system has been greatly helped by genetic analysis. Over 50 genes have been identified by the isolation of specific mutants.
- (1) defects in the production of flagellin (the protein subunit of which the flagella are composed) or in the assembly of the flagella; both lead to non-flagellated cells which are non-motile;
- (2) other flagellar defects that result in flagella that are unable to rotate (these cells are also nonmotile);

Cont…

- (3) defects in the control of rotation, leading to cells that tumble excessively or rarely, or general defects in chemotaxis, so that the cells have normal motility but cannot respond to any stimulus; and
- (4) specific chemotaxis defects, so that the cells can respond to some chemicals but not to others. The genetic techniques summarized earlier, coupled with analysis of the phenotypes involved, enables the establishment of the identity of the genes involved. For example, analysis of specific chemotaxis mutants leads to the identification of the receptors that sense the presence of a specific stimulus.

5) Cell differentiation

- When some bacteria are starved, they are able to respond by producing a resistant endospore. Sporulation has been most extensively studied in Bacillus subtilis and Sporulation in Bacillus takes about 7 h, and can be divided into a number of stages (see Figure 3.7) on the basis of the morphological changes that can be observed microscopically.
- Mutant strains of B. subtilis that are unable to form spores are divided into categories according to the stage at which development of the spores is arrested.

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- Thus any gene in which mutation results in sporulation being blocked at stage II (and unable to progress to stage III) is referred to as spoII, while those in which sporulation proceeds to stage III before stopping are known as spoIII mutants and so on.
- At each stage there are a number of genes that are essential if the process is to continue to the next stage which can be distinguished by complementation and recombination analysis; the different genes are therefore denoted by the addition of further letters and numbers.

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- For example, one class of spoII mutants was designated spoIIA on the basis of genetic analysis. The gene concerned was isolated by cloning a DNA fragment that could complement a spoIIA mutant.
- The sequence of this fragment showed that it contained an operon of three genes, which were designated spoIIAA, spoIIAB and spoIIAC. The sequence of SpoIIAC is similar to that of known sigma (*s) factors, which determine the promoter-specificity of RNA polymerase.* This suggested that SpoIIAC (now known as *sF) is responsible for activating* expression of the forespore genes required in stage III and this was subsequently verified by in vitro tests of purified SpoIIAC.
- The other two spoIIA genes regulate the activity of SpoIIAC so that it only becomes active at the appropriate stage; SpoIIAB is an anti-sigma factor that inhibits SpoIIAC (*sF), while SpoIIAA is an anti-anti-sigma factor that antagonizes SpoIIAB.*

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- A large number of sporulation genes have been identified; the annotated genome sequence lists well over 100. In some cases, the function of the genes is known and they can be given more descriptive names. For example, spoIIAC is listed as sigF. Analysis of the genome sequence by itself would only have enabled a provisional identification of the function of a handful of these genes.
- The isolation and characterization of a large number of mutants provided the foundation for our understanding of the process of sporulation and in particular the regulation of the process, However, there are still many aspects that are not understood, including the precise function of many of the genes that are known to be essential for sporulation.

a) Sporulation in Streptomyces

- Streptomyces (especially S. coelicolor) produce a different sort of spore that is a dispersal mechanism rather than a survival strategy. These bacteria grow initially as filaments but after a few days they differentiate into aerial mycelia with chains of spores at their ends. Some mutants (designated bld, or bald) are unable to produce aerial mycelia, while another class (whi, or white – since the colonies lack the colour associated with the spores) produce aerial mycelia but no spores.
- .

b) Communication and differentiation

- Some bacteria have more complex life cycles, involving distinct morphological stages (and in some cases multicellular behaviour). These form a useful bridge between the simpler bacterial systems and the complex developmental systems in multicellular organisms.
- One example is the aquatic bacterium Caulobacter crescentus, which exists in two forms, stalked and swarmer cells. The former is attached to a substrate by means of its stalk (Figure). As it grows, a flagellum is formed at the end of the cell opposite to the stalk so that at cell division a new, motile, swarmer cell is liberated – the other cell remaining attached to the substrate.

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- The swarmer cell does not replicate its DNA nor undergo cell division but eventually settles down at a new site, sheds its flagellum and forms a stalk to attach to the new substrate. It has now become another stalked cell which carries out DNA replication and a new cell division cycle.
- This poses some interesting questions about the mechanisms controlling this behaviour, including both the regulation of gene expression in the two types of cell and the reasons for the developmental asymmetry of the replicating cell.

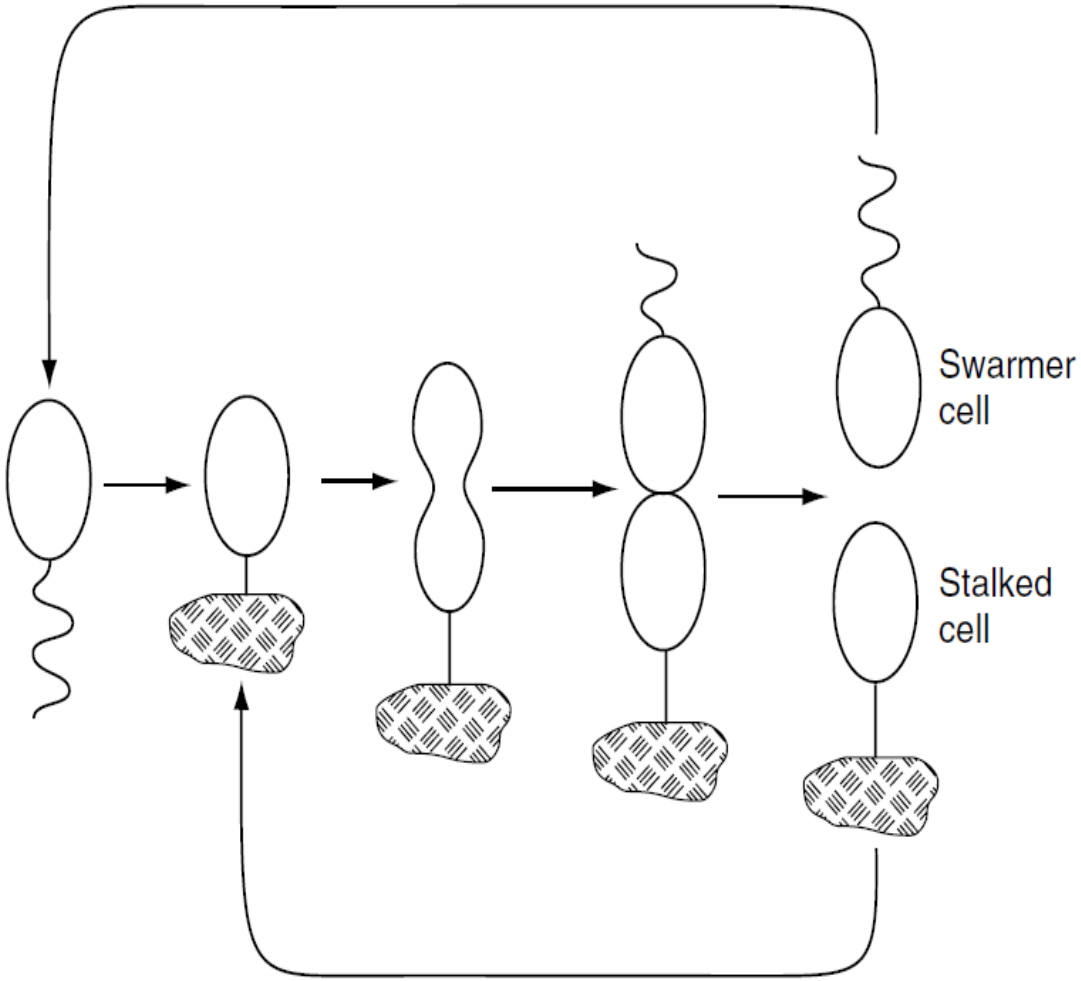

Figure 9.6 Division cycle of *Caulobacter crescentus*. The stalked cell attached to a surface, carries out DNA replication and cell division, producing a motile, non-replicating, swarmer cell. This eventually sheds its flagellum, attaches to a surface and becomes a replicating stalked cell

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- Reporter gene technology and microarray analysis has shown differences in gene expression in the stalked and swarmer cells. One of the regulatory factors involved is a transcriptional activator called FlbD. The active form of FlbD is unequally distributed in the dividing cell, being only found in the swarmer pole. Amongst other things, this activates the genes needed for flagellum production in the swarmer cell. Another bacterium with an interesting life cycle, that includes elements of multicellular behaviour, is the soil bacterium Myxococcus xanthus.
- Under favourable conditions, individual cells swarm over surfaces in a coordinated manner. When food becomes scarce, the individual cells aggregate into a mound (containing some 105 cells) which develops into a fruiting body (Figure) containing a large number of spores (myxospores).
- The myxospores are resistant to drying and enable the bacteria to survive until conditions improve, when they germinate and form individual cells again.

Cont…

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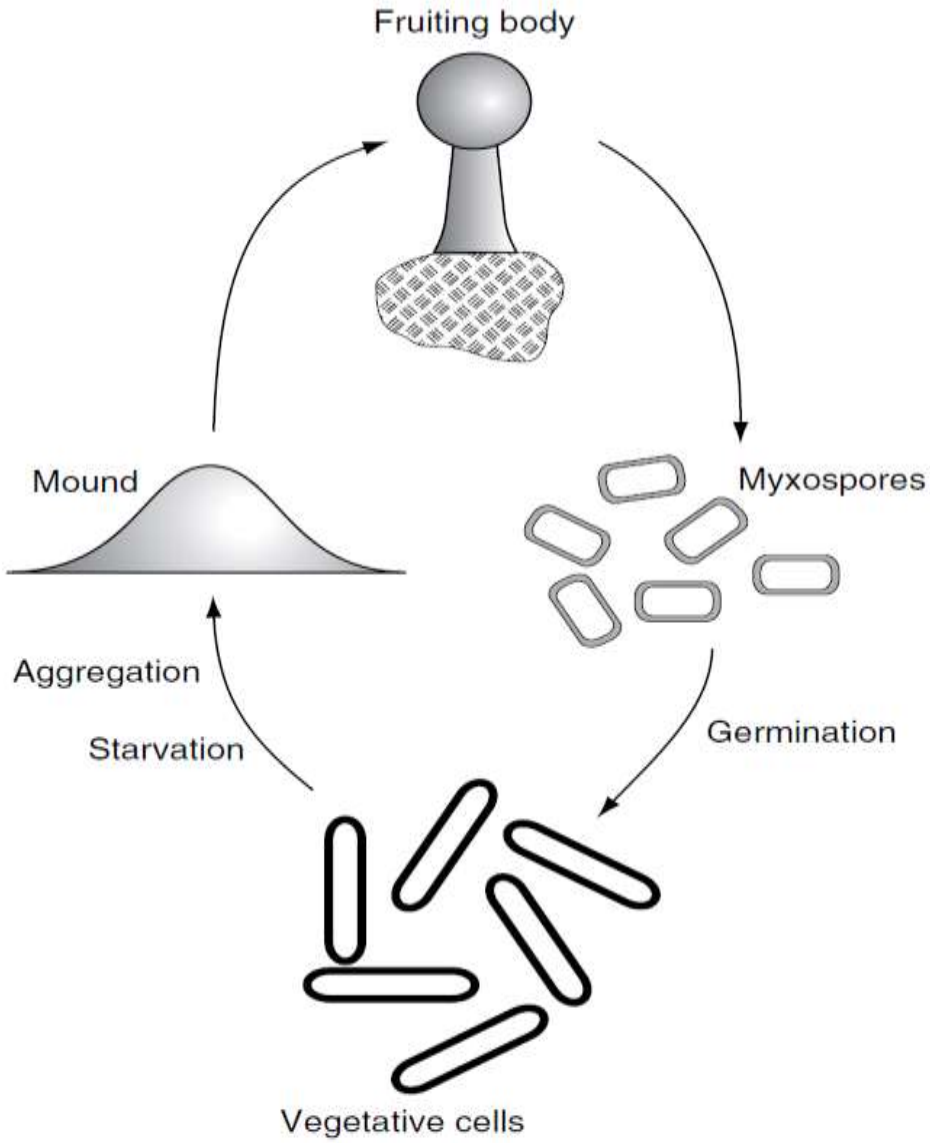

Figure 9.7 Schematic illustration of the life cycle of myxobacteria. This is a generalized and simplified diagram. In different species, the structure of the fruiting body varies substantially, from simpler forms without a stalk to more complex branched structures with multiple sporangia

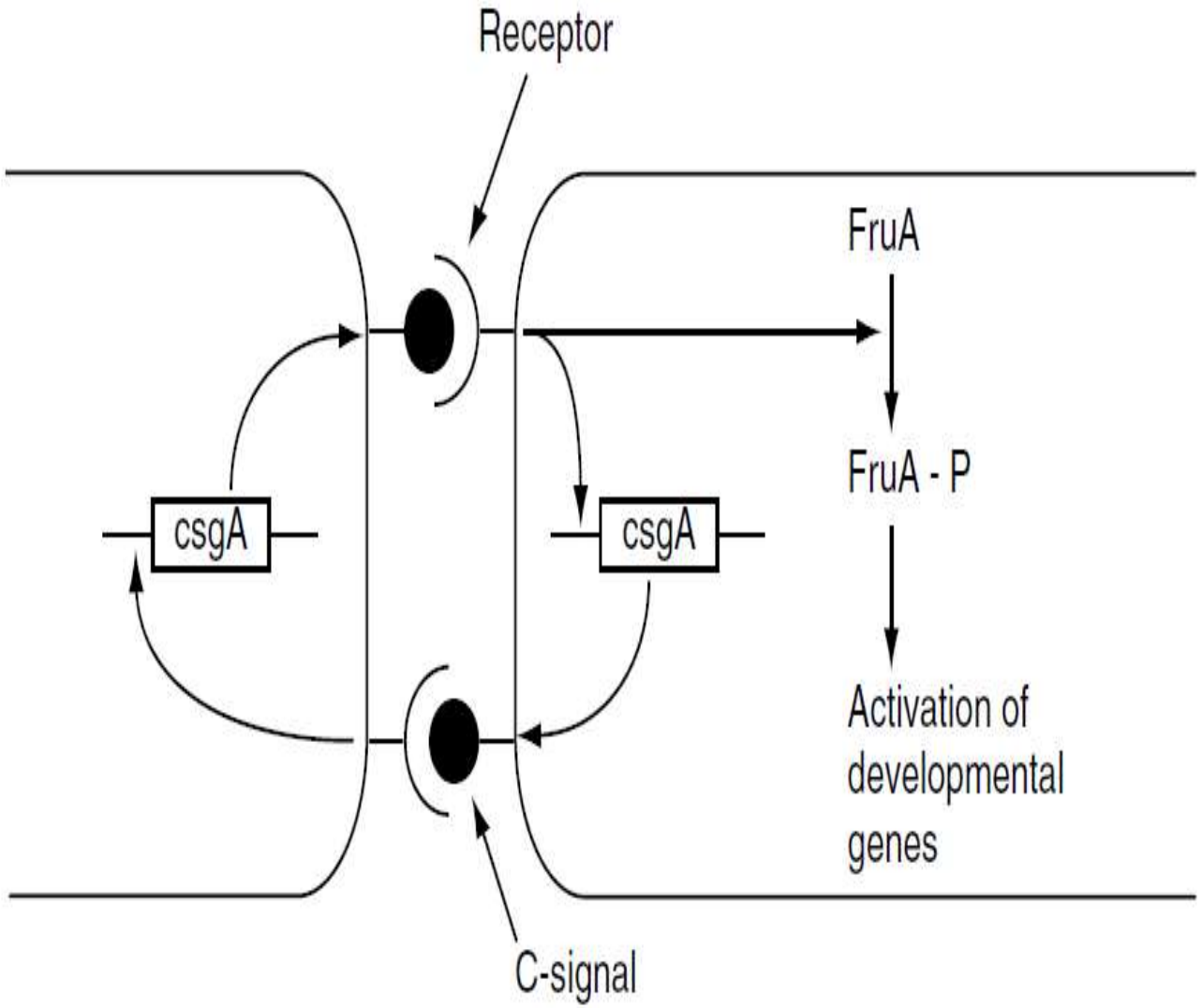

Figure 9.8 Simplified model of some elements of the C-signal pathway in *Myxococcus xanthus*

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- Development from a swarm of individual cells to an aggregate and then on to sporulation is controlled by a series of signals. One of these, the A-signal is a mixture of amino acids that operates as a quorum-sensing mechanism at an early stage of the process.
- Aggregation only occurs if the A-signal reaches a threshold level, thus ensuring that there are enough cells to form a functional fruiting body. Further development is controlled by another gene (csgA) coding for the C-signal, so that csgA mutants are also defective in sporulation. Unlike the A-signal, which is secreted, the C-signal requires cell contact, since it is a cell-surface protein which interacts with a surface receptor on another cell (Figure).

Bacterial virulence

1) Wide range mechanisms of bacterial pathogenesis;

- Bacteria exploit a number of common molecular mechanisms to achieve a range of different objectives during infection. This sectiomolecular genetics has been used to uncover these key mechanisms of microbial pathogenicity. From genome sequence information, it is clear that virulence genes often occur in clusters and that these regions are absent from closely related non-pathogenic bacteria.
- Furthermore, based on the%GCcontent of these regions compared to the rest of the genome, it became apparent that these large sections (e.g. 30–50 kb) of DNA had been acquired from other organisms.

Cont…

- Together, these observations gave rise to the concept of pathogenicity islands which suggests that bacteria can acquire, en masse, complete systems that expand their ability to exploit different host environments.
- Although not expected at the time, a number of related studies on the plant pathogen Pseudomonas syringae were to have dramatic implications for our understanding of pathogenicity. In the mid-1980s transposon mutagenesis was used to identify a cluster of genes which were essential for the generation of plant disease symptoms.

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- The importance of this region was confirmed when the gene cluster was cloned into non-pathogenic bacteria using shuttle vectors and was shown to confer upon these avirulent cells the ability to generate specific disease symptoms.
- In the early 1990s it became clear that similar gene clusters existed in many other bacterial pathogens and that some of the proteins encoded by these gene clusters resembled parts of the machinery for exporting the bacterial flagellum. This information gave rise to the concept that a common secretion pathway existed in these bacterial pathogens.

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- It is now known that these regions encode a specialized secretory apparatus called a type III secretion system.These comprise at least 20 proteins and are the most complex transport systems known in bacteria. They are often referred to as ‘molecular syringes’ as they enable Gram-negative bacteria to secrete and inject effector proteins directly into the cytosol of eukaryotic host cells.
- The injected proteins can redirect the normal cellular signal transduction pathways and can result in disarmament of host immune responses or in cytoskeletal reorganization to establish pathways for bacterial colonization.

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- Much of the type III secretory apparatus is conserved between distantly related pathogens (although the effector proteins differ).
- Thus the same general bacterial pathogenicity mechanism is involved in a multitude of diseases from bubonic plague in humans to southern wilt in tomato plants. *Yersinia* spp., for example, inject at least three different effector molecules to destroy key functions of immune cells. The genes encoding type III secretion systems, including effector proteins and structural proteins, are clustered together as another example of pathogenicity islands that have been transferred between bacterial species.

2) Detection of virulence genes

- Many bacterial pathogens have separate free-living and pathogenic life cycles, so they encounter very different environments and require very different functions for survival. As a consequence, pathogens must be able to recognize signals in the host that convey the need to express virulence genes.
- The expression of certain virulence gene functions in Shigella, for example, is triggered at body temperature (37 8C) but not at environmental temperatures (< 30 C). Therefore, if the genes which are only expressed during infection can be identified, then the key virulence traits can also be identified. Reporter genes have been especially useful for this purpose.

a) In Vivo Expression Technology (IVET)

- One of the most widely used methods of using reporters to identify virulence genes is known as In Vivo Expression Technology (IVET).
- This is a technique that selects bacterial promoters which are only expressed in the host and which thus drive the expression of virulence traits. In most examples of IVET, random fragments of DNA from the bacterial host are inserted adjacent to a promoterless reporter gene whose product confers a phenotype that can be positively selected for in the host. For example, a purA mutant of Salmonella typhimurium is unable to survive in an animal model because purine biosynthesis is essential for Salmonella in this environment.

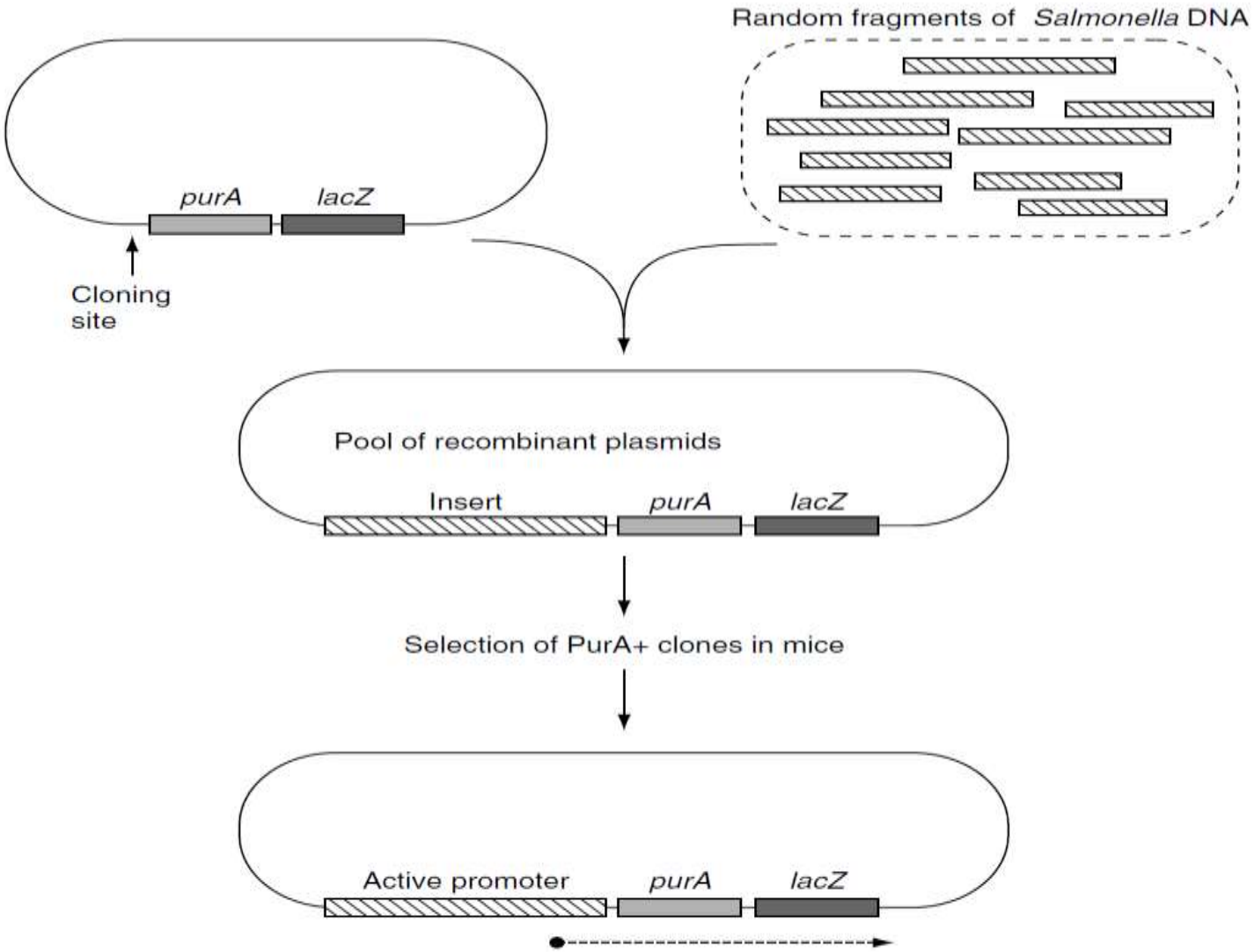

Figure 9.9 *In vivo* expression technology (IVET). The plasmid vector contains promoterless *purA* and *lacZ* genes; expression of these genes is dependent on insertion of a DNA fragment with promoter activity. Infection of mice with a pool of *Salmonella* containing recombinant plasmids is selective for PurA$^+$ clones which contain a promoter that is active under these conditions

Cont…

- This defect can be complemented by a plasmid carrying a functional purA gene, but the purA gene on the plasmid will only be expressed if a promoter is inserted which is functional in vivo (Figure 9.9).
- (The construct shown also contains a lacZ gene whose expression is also controlled by the inserted promoter so that constitutively expressed promoters can be identified and excluded).

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- When animals are infected with a pool of clones each carrying a different DNA fragment, only those clones containing an active promoter are able to survive and to be recovered from the animals, thus providing direct selection for those promoters which are active during infection.
- Identification of these promoter fragments then leads to identification of the genes that are normally regulated by them, which are likely to include genes which are essential for the virulence of Salmonella.

3) Signature tagged mutagenesis

- As shown previously, the classical approach to identifying the genes responsible for a given phenotype would be to generate mutants that are defective in that phenotype. For virulence genes, the phenotyinability to survive in an in vivo model for the disease. Isolation of the mutants cells will prove to be difficult as they are the very cells which do not survive. To circumvent this obstacle, a procedure called signature tagged mutagenesis (STM) has been invented.
- This is essentially a negative selection technique derived from transposon mutagenesis in which each individual mutant is labelled with a unique DNA signature.

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- An overview of STM is given in Figure 9.10. A library of mutants is made for the bacterium of interest and each mutated gene tagged with a section of DNA containing a unique central region and two flanking arms which share their sequence with all of the other tags.
- The key to STM is that each individual mutant can be distinguished from every other mutant based on the possession of its own unique tag. The mutants are then stored individually in ordered arrays and DNA from each one spotted onto a membrane in an grid-like manner.
- The mutants are then pooled and inoculated into a relevant animal model and the bacteria that are able to survive and establish infection are recovered.

Cont…

- The mutants are then pooled and inoculated into a relevant animal model and the bacteria that are able to survive and establish infection are recovered. PCR is then used to amplify all the tags present in the recovered bacteria and the mixed product is used to probe the gridded membrane. Mutants that fail to survive in the infected animal (those which are defective in virulence) can be identified since their tags will not be present in the output pool. They can be recovered from the original stored arrays for further study.
- This system has been widely used to identify novel virulence factors that are involved in colonization, immune system evasion and attachment to human cells in a number of bacterial pathogens.

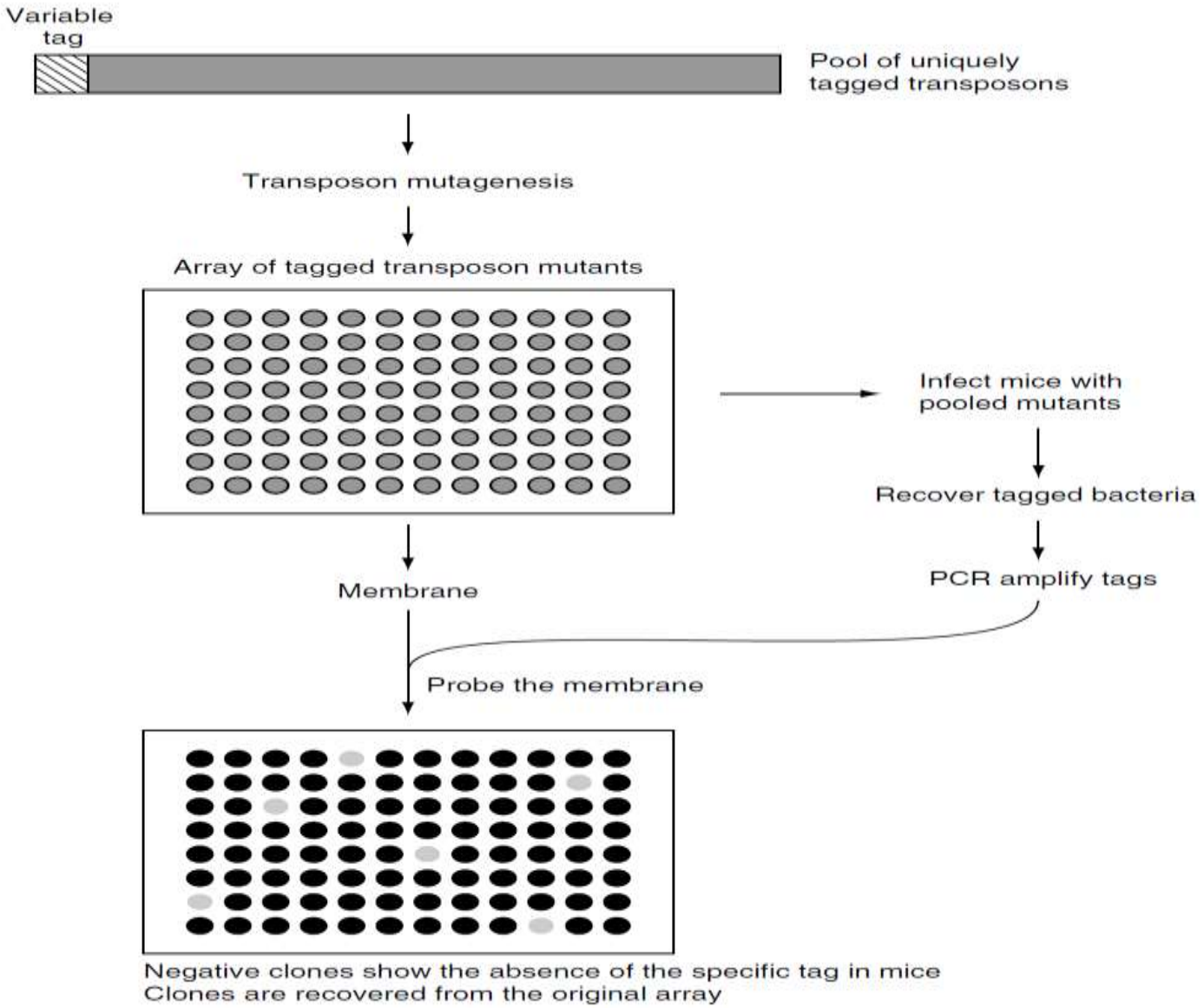

Figure 9.10 Signature tagged mutagenesis. This procedure uses transposon mutagenesis to inactivate genes that are needed for infection of mice and provides a method of identifying those genes

Specific mutagenesis

- Conventional mutational techniques have been considered – producing mutants with an altered phenotype and then identifying the genes affected and determining their functions. These techniques can be complemented by the use of recombinant DNA technology. In contrast to conventional genetics, the recombinant DNA approach starts with an hypothesis that a specific gene is involved.
- This gene is then modified or deleted and the resultant phenotype characterized. So, while conventional genetics starts with the phenotype and works towards identifying the nature and function of the genes involved, the molecular approach starts by altering the gene and works towards an analysis of the phenotype.

1) Gene replacement

- A key technique for determining the function of a specific gene is to inactivate it by a process known variously as gene replacement, allelic replacement or gene knock-out. Essentially, this uses homologous recombination to remove all or part of a specific gene or to replace it with an altered or inactivated gene.
- An example of how this can be done is shown in Figure 9.11. In this case, a plasmid has been constructed in which the central part of the cloned gene has been removed and replaced by an antibiotic resistance gene (aph, aminoglycoside phosphotransferase which confers resistance to kanamycin). The kanamycin resistance gene is flanked by regions of DNA that are the same as those in the host strain and it is within those regions that homologous recombination will occur.

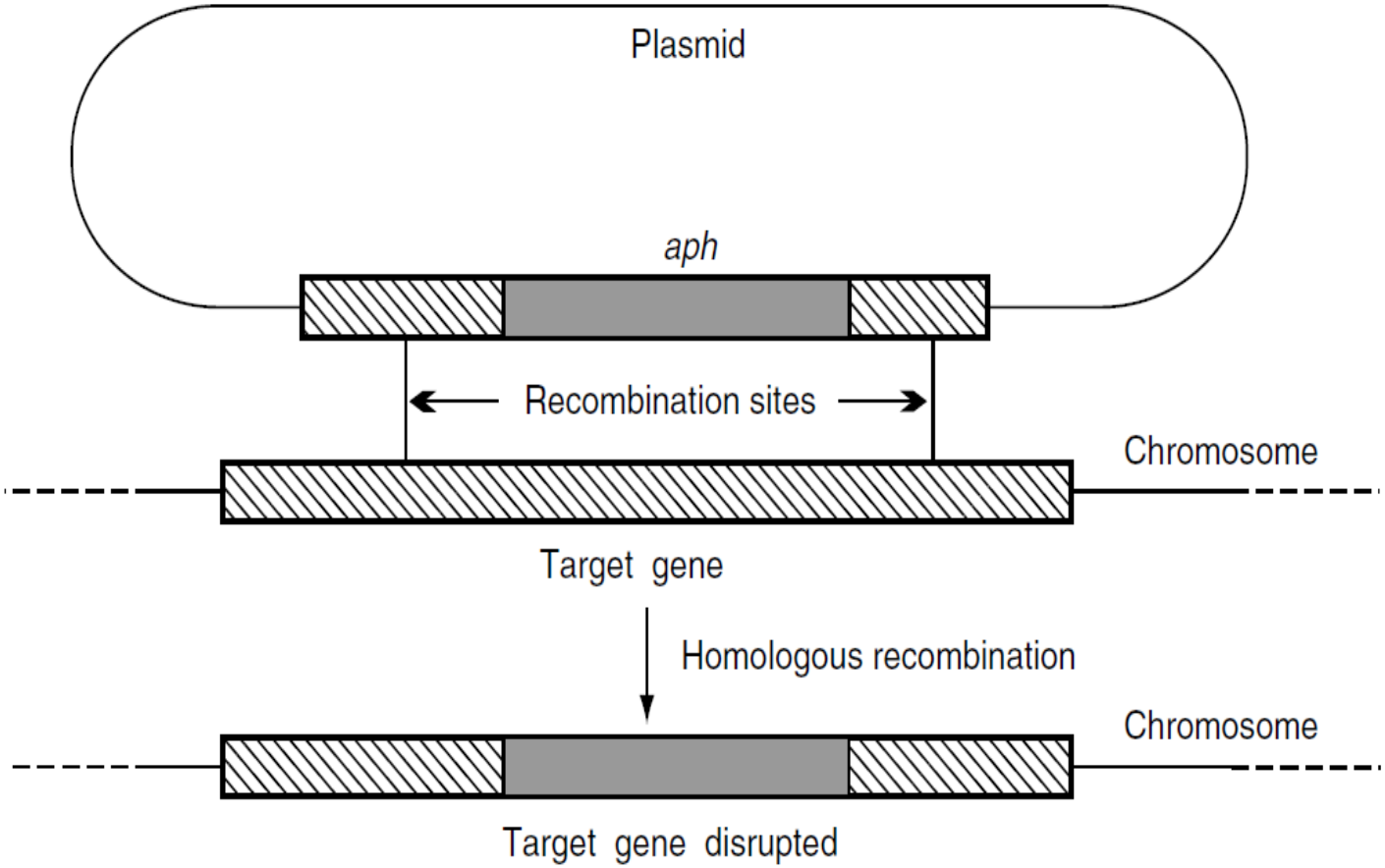

Figure 9.11 Gene disruption by allelic replacement. Homologous recombination between the disrupted cloned gene and the target gene on the chromosome leads to inactivation of the target gene. *aph*, aminoglycoside phosphotransferase (kanamycin resistance)

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- Note that two recombination events (a double crossover) are needed for gene replacement. Recombination at a single site (a single crossover) will merely integrate the plasmid into the chromosome. Additional techniques are needed to ensure that a double crossover is achieved.
- The plasmid that is used is one that cannot replicate in the chosen host cell. Thus after transformation, selection for kanamycin resistance will isolate cells in which the aph gene has been inserted into the chromosome by homologous recombination with the target gene, thereby inactivating that gene.

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- Tests can be carried out to ascertain whether the expected phenotype is produced. For example, if it has been assumed that the target gene is necessary for survival and growth within macrophages, then testing the mutant for a deficiency in this respect will confirm or deny the assumption. The results do not necessarily prove the case absolutely.
- Genes interact in many ways and knocking out one gene may have indirect effects on others. In particular, if the target gene is part of an operon, the gene knockout may affect the expression of other genes within the operon (in other words, the mutation may be polar). This possibility can be partly eliminated by the use of complementation.

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- A plasmid carrying the wild-type gene (in this case using a plasmid that can replicate in this host) can be introduced into the mutant and if the original gene knock-out was responsible for the observed effect (e.g. loss of ability to grow within macrophages), then the introduced plasmid will restore the original, wildtype phenotype.
- Successful complementation therefore supports the contention that the product of this gene is necessary for survival in macrophages. But it needs to be said that it still does not finally establish a direct role for the gene product – for example, it could function by altering the expression of other genes. In this way, the significance of a specific amino acid in the activity of an enzyme for example, can be tested by replacing it with a series of different residues and determining the activity of the product.

2) Antisense RNA

- One problem with gene knock-outs is that if the gene is essential for the growth of the cell in the laboratory, then the complete loss of that gene would be lethal and therefore no recombinants would be obtained.
- It may therefore be useful to partially reduce the expression of the gene or the functionality of its product – thus leaving enough activity to cope with the comfortable conditions of normal laboratory growth but not enough to deal with the stress conditions that may be imposed on it subsequently. One alternative strategy is to use antisense RNA.

Cont…

- For this purpose, part of the gene would be cloned in an expression vector, so that it is transcribed from a promoter on the vector, but the insert would be deliberately put in the wrong orientation.
- The insert will therefore be transcribed in the opposite direction from normal – or in other words, the opposite strand will be transcribed. The RNA produced (the antisense RNA) will be complementary to the normal mRNA and will pair with it to produce a double-stranded RNA molecule.
- This may interfere with translation of the mRNA and thus reduce the level of the protein that is made. Using a stronger or a weaker promoter (or even better, using a promoter that can be switched on and off) will lead to different amounts of the antisense RNA being made and hence will alter the extent of the reduction in the amount of the protein product formed.

Taxonomy, evolution and epidemiology

1) Molecular taxonomy;

- It is possible to distinguish between even closely related organisms using a range of biochemical tests, but this approach does not necessarily give an accurate picture of the true taxonomic or evolutionary relationship between different organisms.
- One simple molecular characteristic that is used to classify bacteria is the base composition of the DNA, defined as the number of guanine and cytosine residues as a percentage of the total number of bases (%GC).
- It is not necessary to sequence the genome to determine this value.

Cont…

- The %GC can be determined using physical techniques. The base composition of bacterial DNA varies widely from one species to another – over a range of 20–80 per cent – but closely related organisms tend to have similar DNA base compositions.

- If this concept is applied to bacterial taxonomy, it is sometimes found that organisms which are otherwise quite similar have quite different DNA base composition. For example the genera Staphylococcus and Micrococcus are morphologically similar Gram-positive cocci (although they can be distinguished biochemically). However Micrococcus has a high GC content (about 70 per cent GC), while Staphylococcus DNA has a low proportion of GþC (30–39 per cent).

Cont…

- Ribosomal RNA sequencing is a much more powerful technique. The ribosomal RNA genes are very highly conserved, being remarkably similar in all bacteria, and yet there are small variations in the sequence from one species to another. These variations (most commonly in the 16S rRNA) not only distinguish between species but also indicate the degree of difference. In other words, by counting the number of bases that are different in two species a measure of the evolutionary distance that separates them can be calculated.
- If a number of such sequences is compared, a phylogenetic tree can be constructed which will show a possible route by which these species have diverged from a common ancestor. A simple example (with a much shorter sequence than would be used in practice) is shown in Figure 9.12.

Cont…

- The sequence of organism A is more similar to B than it is to C or D for example and this is reflected in the arrangement of the tree.
- A word of caution: construction of a phylogenetic tree is much more complicated than this simple description and many trees can be drawn from a single set of data. The computer produces the best fit, but it is only a model and does not necessarily reflect the true evolution of the organisms involved.
- Cloning the ribosomal RNA genes to do this is not necessary. The variation in the 16S (or 23S) rRNA gene is not evenly spread across the gene.

Cont…

- Some regions are particularly highly conserved, so a pair of PCR primers can be used which recognize conserved sequences on either side of a variable region and amplify the region which contains differences. This amplified product can then be sequenced.
- The degree of conservation is such that the same pair of primers can be used for any organism, without knowing anything about it. The sequence obtained can then be compared with sequences of rRNA from known organisms and thus the identity of the unknown bacterium and its relationship to known species can be determined, at least provisionally.

Organism	Sequence
A	C A U A G A C C U G A C G C C
B	C C U A G A G C U G G C G C G
C	C G A A G A G G U G G C G C C
D	G C U A G A A G U G G C G G C

Only a short sequence is shown as an example
In practice much longer sequences would be used

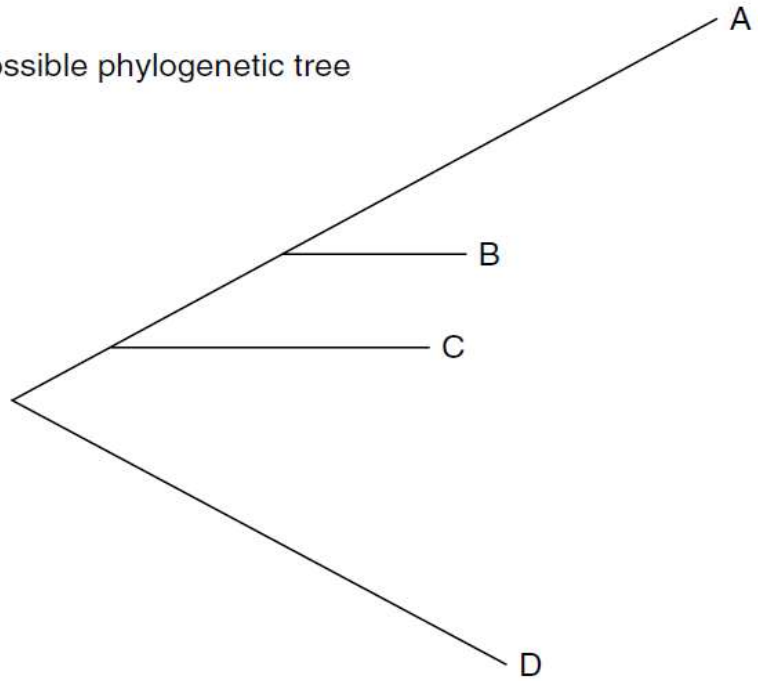

Figure 9.12 Construction of a phylogenetic tree from sequence data. This illustrates the principle only. In practice a much longer sequence would be used and extensive computer analysis is required to test the many possible trees that can be drawn

Cont…

- The power of PCR to amplify minute amounts of DNA means that the bacterium in question does not have to be cultured. This is significant as standard bacteriological techniques are designed to culture certain bacteria, especially medically important pathogens.
- The range of bacteria that can be isolated can be extended by using different media and different growth conditions. But however wide the range of conditions used, there will still be some bacteria – often a substantial majority – that are unable to grow. Applying PCR to such a sample, using primers directed at the 16S rRNA gene, will produce a very wide range of amplified products.

Cont…

- Cloning this mixture of products, rather like constructing a gene library,enables each one to be isolated and sequenced so that the bacteria present in the sample can be identified (within the limitations of the known sequences in the database).
- For example, the bacterial flora of the human colon has been extensively investigated using cultural techniques and its constituent bacteria were thought to have been thoroughly characterized. However, the genotypic approach described above showed that 76 per cent of the 16S rRNA genes generated did not correspond to known organisms and were clearly derived from hitherto unknown and uncultured bacterial species.

a) Diagnostic use of PCR

- Traditional methods for the detection and identification of bacteria rely on growing the organism in pure culture and identifying it by a combination of staining methods, biochemical reactions and other tests. This applies equally to detection of environmental organisms (in soil or water), bacteria in food (including milk and drinking water) or pathogens in samples from patients with an infectious disease.
- However these methods are slow, requiring at least 24 h or several weeks for slow-growing organisms such as Mycobacterium tuberculosis. In addition, there are some bacteria, such as Mycobacterium leprae (the causative agent of leprosy) that still cannot be grown in the laboratory.

Cont…

- In principle, gene probes could be used to provide quicker results by directly detecting the presence of specific DNAin the specimen. However, this only works if the bacteria present are plentiful. Gene probes are not sensitive enough to detect the small numbers of organisms that may be present, and significant, in such specimens.
- This provides greatly enhanced sensitivity, being capable (in theory) of detecting a single organism. In order to apply this to the detection of a specific species, it is necessary to know the sequence of a gene that is characteristic of that species – that is, it is always present (and the sequence is conserved) in that species, but is absent or significantly different in other bacteria.

Cont…

- A pair of PCR primers can then be designed which will anneal to this target sequence so that PCR will amplify a DNA fragment that can be easily detected.
- Other bacteria, lacking the specific binding sites for those primers, will not give an amplified product. In a research laboratory the amplified product (amplicon) would commonly be detected by gel electrophoresis, sometimes combined with Southern blotting and hybridization with specific gene probes to increase the sensitivity and specificity of the procedure. The commercial kits that are now available for detection of some bacterial pathogens (some using forms of gene amplification that are distinct from PCR) use other, quicker, ways of detecting product amplification.
- A technique known as real-time PCR which produces results more rapidly than gel electrophoresis and has the additional advantage of quantifying the target present in the sample.

2) Molecular epidemiology

- Epidemiology is the study of the occurrence and distribution of diseases. By identifying the source of infection, the measures necessary to control an outbreak can be determined. The microbiology laboratory contributes to this effort by identifying the pathogen and determining the strain involved (strain typing).
- There are a wide variety of methods available for typing different bacterial species. Serotyping, using variable antigens, molecular typing
- One of the most widely applicable of such methods is restriction fragment length polymorphism (RFLP). When a gene probe is hybridized to a Southern blot of restriction enzyme-digested DNA from different strains, the size of the fragment(s) detected may vary from one strain to another.

Cont…

- This effect can arise from point mutations that remove (or create) restriction sites or from the insertion or deletion of DNA fragments in the region detected by the probe (see Figure. Insertion sequences can be extremely useful for this purpose, since there are often multiple copies of the element in a strain (giving rise to a number of bands on the Southern blot) and also because the site of insertion in the chromosome is often highly variable .
- One element that is widely used for epidemiological purposes is the insertion sequence IS6110 in M. tuberculosis. With this probe, similar patterns (fig.) are obtained only with strains from the same outbreak.

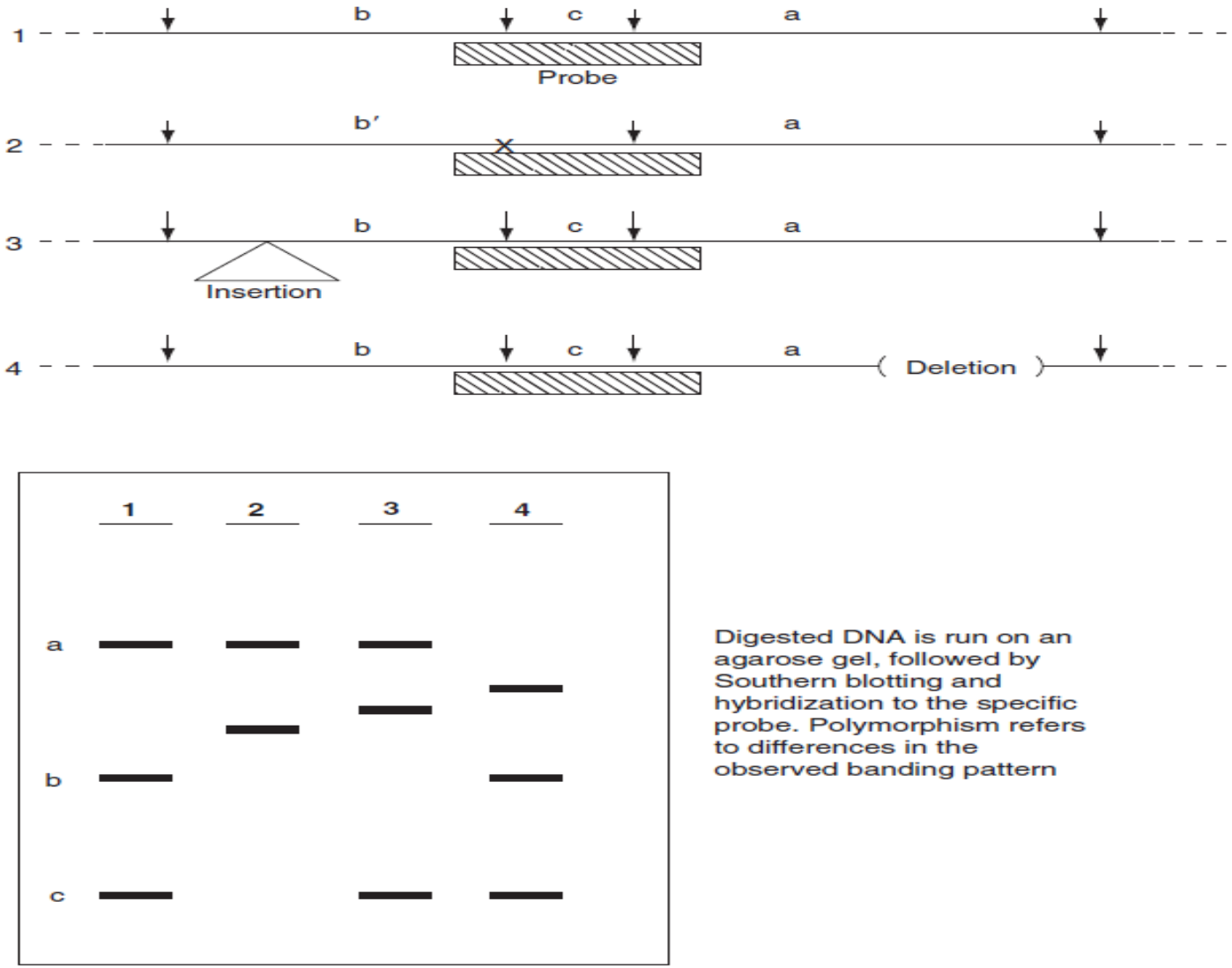

Figure 9.13 Restriction fragment length polymorphism (RFLP). The diagram shows a region of the chromosome that hybridizes to a specific probe and some of the possible reasons for polymorphism in the Southern blot pattern: (1) The 'original' sequence, in which the probe detects three DNA fragments labelled a,b and c. (2) Loss of a restriction site by mutation results in fragments b and c being replaced by a single larger fragment. (3) An insertion within fragment b changes its size, without altering the number of fragments. (4) A deletion within fragment a reduces its size

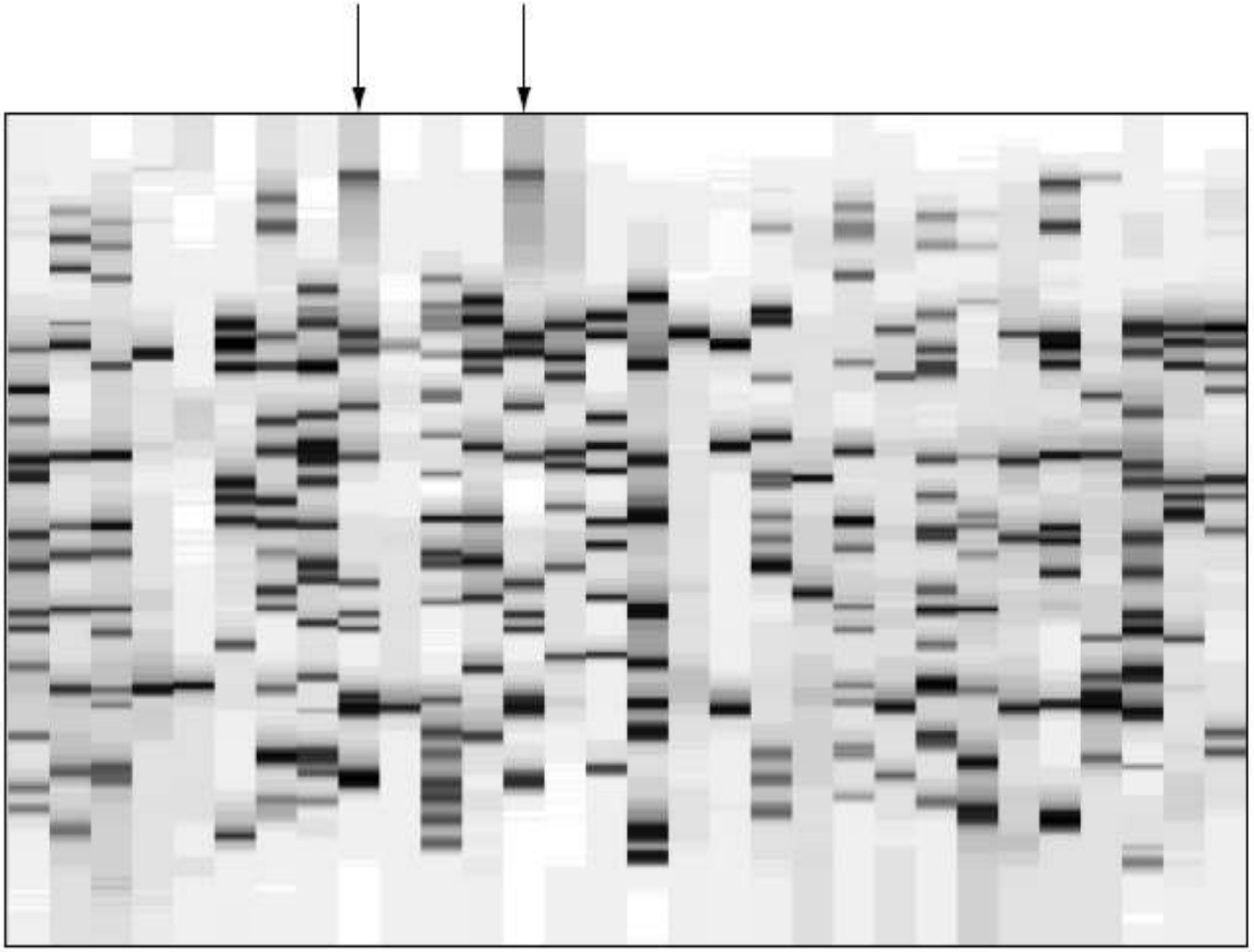

Figure 9.14 Fingerprinting of *Mycobacterium tuberculosis* using IS*6110*. Isolates from cases with a common source of infection show identical patterns (arrowed)

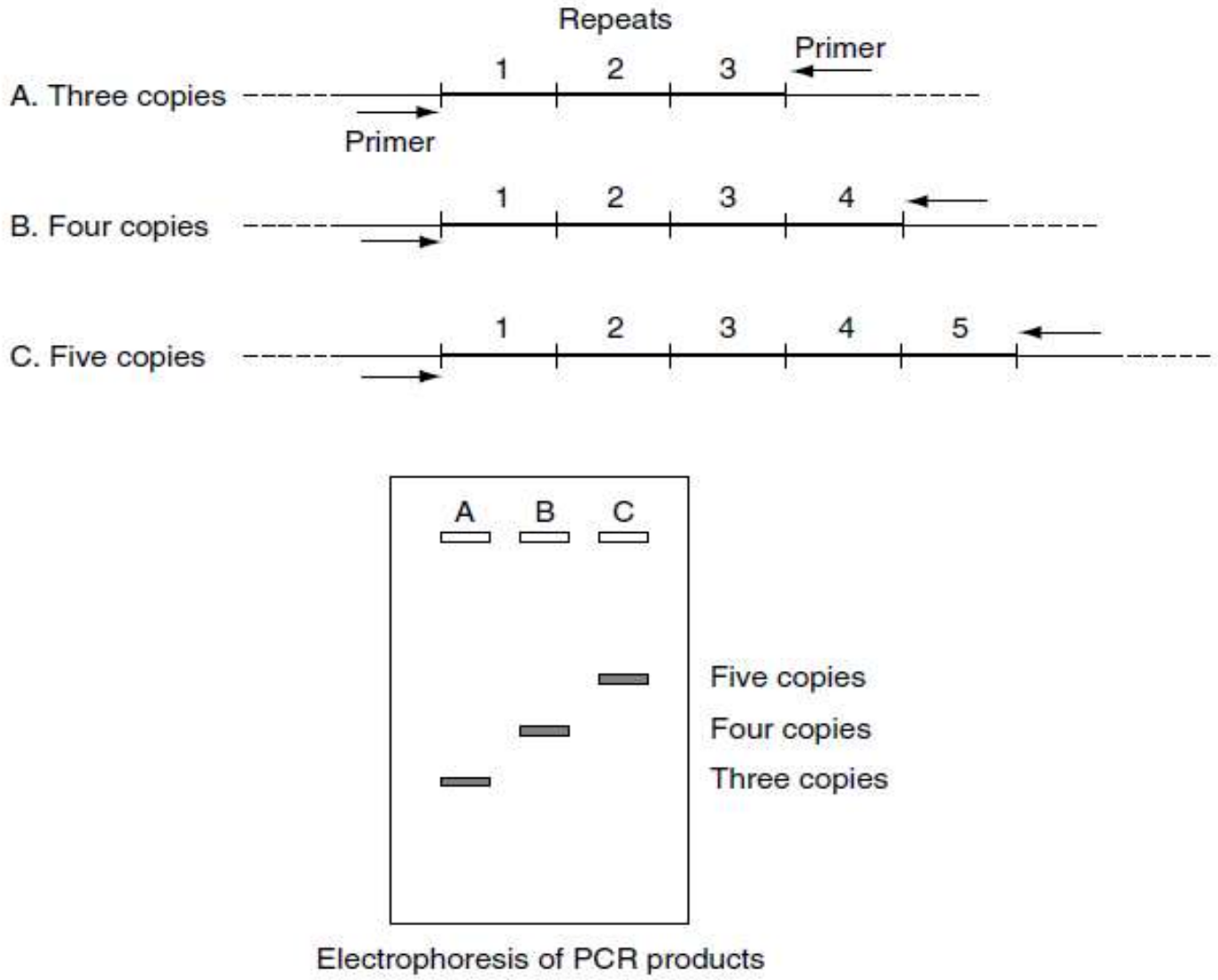

Figure 9.15 Variable Number Tandem Repeats (VNTR). PCR amplification of a region containing tandem repeats can be used for bacterial typing by determining the number of copies of the repeated sequence

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Thank you