

1.1

Microorganisms

Key Points

- Bacteria can be classified according to their staining by the Gram stain (Gram positive, Gram negative, and mycobacteria) and their shape.
- Most bacterial (prokaryotic) cells differ from mammalian (eukaryotic) cells in that they have a cell wall *and* cell membrane, have no nucleus or organelles, and have different biochemistry.
- Bacteria can be identified by microscopy, or by using chromogenic (or fluorogenic) media or molecular diagnostic methods (e.g. real-time polymerase chain reaction (PCR)).
- Bacterial resistance to an antibacterial agent can occur as the result of alterations to a target enzyme or protein, alterations to the drug structure, and alterations to an efflux pump or porin.
- Antibiotic stewardship programmes are designed to optimise antimicrobial prescribing in order to improve individual patient care and slow the spread of antimicrobial resistance.

1.1.1 Classification

There are two basic cell types: prokaryotes and eukaryotes, with prokaryotes predating the more complex eukaryotes on earth by billions of years. Bacteria are prokaryotes, while plants, animals, and fungi (including yeasts) are eukaryotes. For our purposes in the remainder of this book, we will further subdivide bacteria into Gram positive, Gram negative, and mycobacteria (we will discuss prokaryotic cell shapes a little later).

As you are probably already aware, we can use the Gram stain to distinguish between groups of bacteria, with Gram positive being stained dark purple or violet when treated with Gentian violet then iodine/potassium iodide (Figures 1.1.1 and 1.1.2). Gram negative bacteria do not retain the dark purple stain, but can be visualised by a counterstain (usually eosin or fuchsin, both of which are red), which does not affect the Gram positive cells. Mycobacteria do not retain either the Gram stain or the counterstain and so must be visualised using other staining methods. Hans Christian Joachim Gram developed this staining technique in 1884, while trying to develop a new method for the visualisation of bacteria in the sputum of patients with pneumonia, but

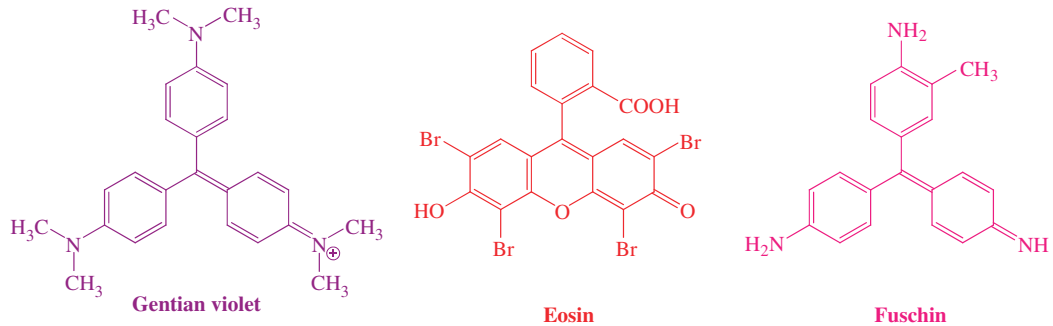


Figure 1.1.1 Dyes used in the Gram stain

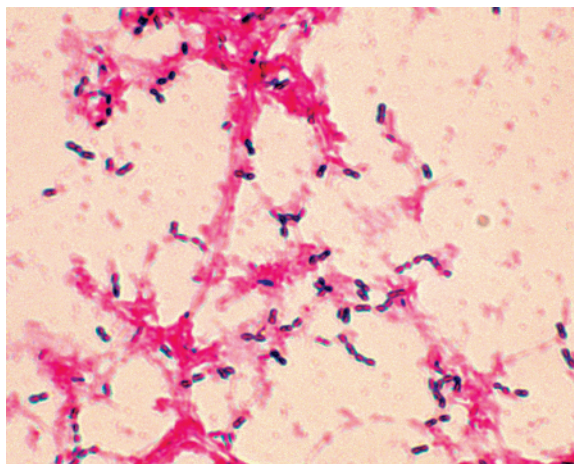


Figure 1.1.2 Example of a Gram stain showing **Gram positive** (*Streptococcus pneumoniae*) and **Gram negative** bacteria (Image courtesy of Public Health Image Library, Image ID 2896, Online, [<http://phil.cdc.gov/phil/home.asp>], last accessed 26th March 2012).)

the mechanism of staining, and how it is related to the nature of the cell envelopes in these different classes of bacteria, is still unclear.

Some of the Gram positive and Gram negative bacteria, as well as some mycobacteria, which we shall encounter throughout this book, are listed in Table 1.1.1.

1.1.2 Structure

The ultimate aim of all antibacterial drugs is selective toxicity – the killing of pathogenic¹ bacteria (bactericidal agents) or the inhibition of their growth and multiplication (bacteriostatic agents), without affecting the cells of the host. In order to understand how antibacterial agents can achieve this desired selectivity, we must first understand the differences between bacterial (prokaryote) and mammalian (eukaryote) cells.

¹ 'Pathogenic' means 'disease-causing'.

Table 1.1.1 Examples of Gram positive and Gram negative bacteria, and mycobacteria

| Gram positive | Gram negative | Mycobacteria |
|--|---------------------------------|--|
| <i>Bacillus subtilis</i> | <i>Burkholderia cenocepacia</i> | <i>Mycobacterium africanum</i> |
| <i>Enterococcus faecalis</i> | <i>Citrobacter freundii</i> | <i>Mycobacterium avium</i> complex (MAC) |
| <i>Enterococcus faecium</i> | <i>Enterobacter cloacae</i> | <i>Mycobacterium bovis</i> |
| <i>Staphylococcus epidermis</i> | <i>Escherichia coli</i> | <i>Mycobacterium leprae</i> |
| <i>Staphylococcus aureus</i> | <i>Morganella morganii</i> | <i>Mycobacterium tuberculosis</i> |
| Meticillin-resistant <i>Staphylococcus aureus</i> (MRSA) | <i>Pseudomonas aeruginosa</i> | |
| <i>Streptococcus pyogenes</i> | <i>Salmonella typhimurium</i> | |
| <i>Listeria monocytogenes</i> | <i>Yersinia enterocolitica</i> | |

The name ‘prokaryote’ means ‘pre-nucleus’, while eukaryote cells possess a true nucleus, so one of the major differences between bacterial (prokaryotic) and mammalian (eukaryotic) cells is the presence of a defined nucleus (containing the genetic information) in mammalian cells, and the absence of such a nucleus in bacterial cells. Except for ribosomes, prokaryotic cells also lack the other cytoplasmic organelles which are present in eukaryotic cells, with the function of these organelles usually being performed at the bacterial cell membrane.

A schematic diagram of a bacterial cell is given in Figure 1.1.3, showing the main features of the cells and the main targets for antibacterial agents. As eukaryotic cells are much more complex, we will not include a schematic diagram for them here, and will simply list the major differences between the two basic cell types:

- Bacteria have a cell wall and plasma membrane (the cell wall protects the bacteria from differences in osmotic pressure and prevents swelling and bursting due to the flow of water into the cell, which would occur as a result of the high intracellular salt concentration). The plasma membrane surrounds the cytoplasm and between it and the cell wall is the periplasmic space. Surrounding the cell wall, there is often a capsule (there is also an outer membrane layer in Gram negative bacteria). Mammalian eukaryotic cells only have a cell membrane, whereas the eukaryotic cells of plants and fungi also have cell walls.
- Bacterial cells do not have defined nuclei (in bacteria the DNA is present as a circular double-stranded coil in a region called the ‘nucleoid’, as well as in circular DNA plasmids), are relatively simple, and do not contain

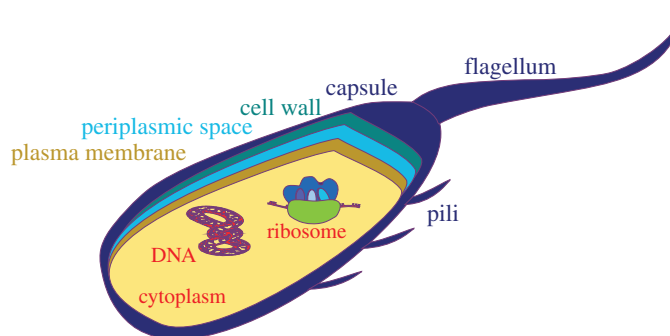


Figure 1.1.3 Simplified representation of a prokaryotic cell, showing a cross-section through the layers surrounding the cytoplasm and some of the potential targets for antibacterial agents

organelles, whereas eukaryotic cells have nuclei containing the genetic information, are complex, and contain organelles,² such as lysosomes.

- The biochemistry of bacterial cells is very different to that of eukaryotic cells. For example, bacteria synthesise their own folic acid (vitamin B9), which is used in the generation of the enzyme co-factors required in the biosynthesis of the DNA bases, while mammalian cells are incapable of folic acid synthesis and mammals must acquire this vitamin from their diet.

Whenever we discuss the mode of action of a drug, we will be focussing on the basis of any selectivity. As you will see from the section headings, we have classified antibacterial agents into those which target DNA (Section 2), metabolic processes (Section 3), protein synthesis (Section 4), and cell-wall synthesis (Section 5). In some cases, the reasons for antibacterial selectivity are obvious, for example mammalian eukaryotic cells do not have a peptidoglycan-based cell wall, so the agents we will discuss in Section 5 (which target bacterial cell-wall synthesis) should have no effect on mammalian cells. In other cases, however, the basis for selectivity is not as obvious, for example agents targeting protein synthesis act upon a process which is common to both prokaryotic and eukaryotic cells, so that in these cases selective toxicity towards the bacterial cells must be the result of a more subtle difference between the ribosomal processes in these cells.

We will now look at these antibacterial targets in detail, in preparation for our in-depth study of the modes of action of antibacterial agents and bacterial resistance in the remaining sections.

1.1.3 Antibacterial targets

1.1.3.1 DNA replication

DNA replication is a complex process, during which the two strands of the double helix separate and each strand acts as a template for the synthesis of complementary DNA strands. This process occurs at multiple, specific locations (origins) along the DNA strand, with each region of new DNA synthesis involving many proteins (shown in *italics* below), which catalyse the individual steps involved in this process (Figure 1.1.4):

- The separation of the two strands at the origin to give a replication fork (*DNA helicase*).
- The synthesis and binding of a short **primer** DNA strand (*DNA primase*).

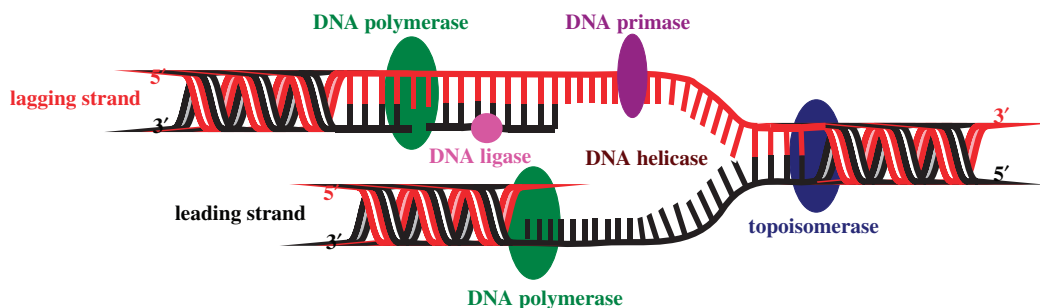


Figure 1.1.4 DNA replication fork (adapted from http://commons.wikimedia.org/wiki/File:DNA_replication_en.svg, last accessed 7 March 2012.)

²Specialised cellular subunits with a specific function.

- DNA synthesis, in which the base (A, T, C, or G) that is complementary to that in the primer sequence is added to the growing chain, as its triphosphate; this process is continued along the template strand, with the new base always being added to the 3'-end of the growing chain (*DNA polymerase*) in the leading strand.
- The meeting and termination of replication forks.
- The proofreading and error-checking process to ensure the new DNA strand's fidelity; that is, that this strand (red) is exactly complementary to the template (black) strand (*DNA polymerase and endonucleases*).

Due to the antiparallel nature of DNA, synthesis of the strand that is complementary (black) to the lagging strand (red) must occur in the opposite direction, and this is more complex than the process which takes place in the leading strand.

DNA helicase is the enzyme which separates the DNA strands and in so doing, as a result of the right-handed helical nature of DNA, produces positive supercoils (knots) ahead of the replication site. In order for DNA replication to proceed, these supercoils must be removed by enzymes (known as topoisomerases) relaxing the chain. By catalysing the formation of negative supercoils, through the cutting of the DNA chain(s) and the passing of one strand through the other, these enzymes remove the positive supercoils and give a tension-free DNA double helix so that the replication process can continue. Type I topoisomerases relax DNA by cutting one of the DNA strands, while, you've guessed it, type II cut both strands (Champoux, 2001). In Section 2.1 we will look at a class of drugs which target the topoisomerases: the quinolone antibacterials, which, as DNA replication is obviously common to both prokaryotes and eukaryotes, must act on some difference in the DNA relaxation process between these cells.

1.1.3.2 Metabolic processes (folic acid synthesis)

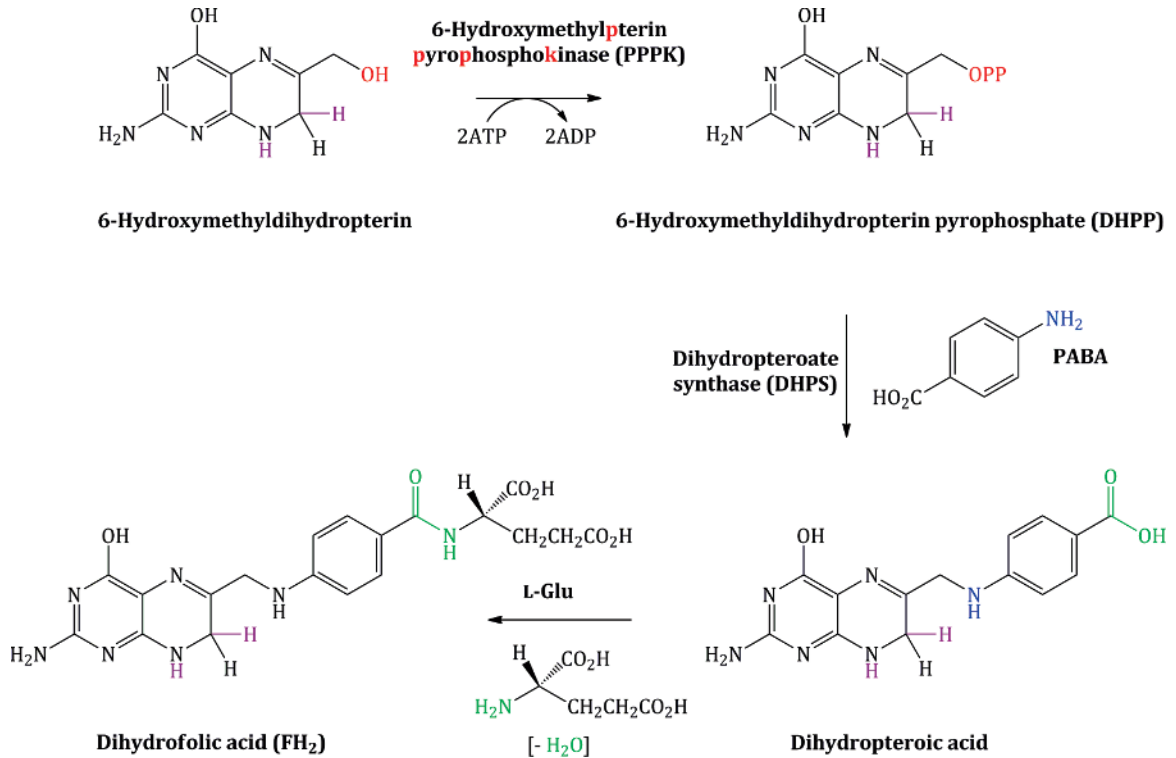
As mentioned above, metabolic processes represent a key difference between prokaryotic and eukaryotic cells and an example of this is illustrated by the fact that bacteria require *para*-aminobenzoic acid (PABA), an essential metabolite, for the synthesis of folic acid. Bacteria lack the protein required for folate uptake from their environment, whereas folic acid is an essential metabolite for mammals (as it cannot be synthesised by mammalian cells and must therefore be obtained from the mammalian diet). Folic acid is indirectly involved in DNA synthesis, as the enzyme co-factors which are required for the synthesis of the purine and pyrimidine bases of DNA are derivatives of folic acid. If the synthesis of folic acid is inhibited, the cellular supply of these co-factors will be diminished and DNA synthesis will be prevented.

Bacterial synthesis of folic acid (actually dihydrofolic acid³) involves a number of steps, with the key steps shown in Schemes 1.1.1 and 1.1.2. A nucleophilic substitution is initially involved, in which the free amino group of PABA substitutes for the pyrophosphate group (OPP) introduced on to 6-hydroxymethylpterin by the enzyme 6-hydroxymethylpterinpyrophosphokinase (PPPK). In the next step, **amide** formation takes place between the free amino group of L-glutamic acid and the carboxylic acid group derived from PABA (Achari *et al.*, 1997).

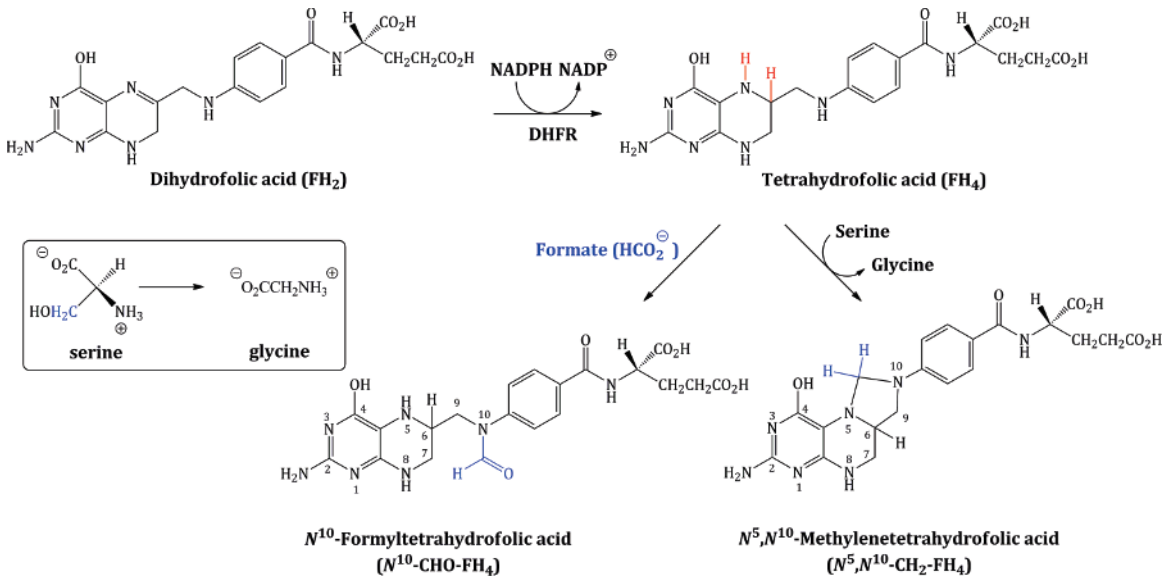
Dihydrofolic acid (FH₂) is further reduced to tetrahydrofolic acid (FH₄), a step which is catalysed by the enzyme dihydrofolate reductase (DHFR), and FH₄ is then converted into the enzyme co-factors *N*⁵,*N*¹⁰-methylene-tetrahydrofolic acid (*N*⁵,*N*¹⁰-CH₂-FH₄) and *N*¹⁰-formyltetrahydrofolic acid (*N*¹⁰-CHO-FH₄) (Scheme 1.1.2).

The tetrahydrofolate enzyme co-factors are the donors of one-carbon fragments in the biosynthesis of the DNA bases. Crucially, each time these co-factors donate a C-1 fragment, they are converted back to dihydrofolic acid, which, in an efficient cell cycle, is reduced to FH₄, from which the co-factors are regenerated. For example, in the biosynthesis of deoxythymidine monophosphate (from deoxyuridine monophosphate), the enzyme thymidylate synthetase utilises *N*⁵,*N*¹⁰-CH₂-FH₄ as the source of the methyl group introduced on to the pyrimidine ring (Scheme 1.1.3).

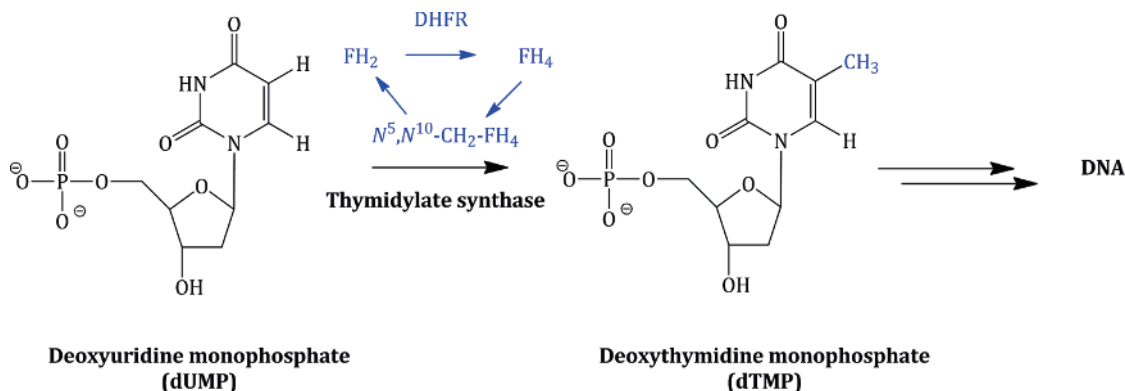
³ The two hydrogens added to folic acid to give dihydrofolic acid are highlighted in purple in Scheme 1.1.1.



Scheme 1.1.1 Bacterial synthesis of dihydrofolic acid



Scheme 1.1.2 Formation of the tetrahydrofolate enzyme co-factors



Scheme 1.1.3 Biosynthesis of deoxythymidine monophosphate (dTMP)

Similarly, N^{10} -CHO-FH₄ serves as the source of a formyl group in the biosynthesis of the purines and, once again, is converted to dihydrofolic acid (which must be converted to tetrahydrofolic acid and then N^{10} -CHO-FH₄, again in a cyclic process).

Cells which are proliferating thus need to continually regenerate these enzyme co-factors due to their increased requirement for the DNA bases. If a drug interferes with any step in the formation of these co-factors then their cellular levels will be depleted and DNA replication, and so cell proliferation, will be halted. In Section 3 we will look more closely at drugs which target these processes: the sulfonamides (which interfere with dihydrofolic acid synthesis) and trimethoprim (a DHFR inhibitor).

1.1.3.3 Protein synthesis

Protein synthesis, like DNA replication, is a truly awe-inspiring process, involving:

- Transcription – the transfer of the genetic information from DNA to messenger RNA (mRNA).
- Translation – mRNA carries the genetic code to the cytoplasm, where it acts as the template for protein synthesis on a **ribosome**, with the bases complementary to those on the mRNA being carried by transfer RNA (tRNA).
- Post-translational modification – chemical modification of amino acid residues.
- Protein folding – formation of the functional 3D structure.

Throughout this process, any error in transcription or translation may result in the inclusion of an incorrect amino acid in the protein (and thus a possible loss of activity), so it is essential that all of the enzymes involved in this process carry out their roles accurately. (For further information on protein synthesis, see Laursen *et al.*, 2005; Steitz, 2008.)

During **transcription**, DNA acts as a template for the synthesis of mRNA (Figure 1.1.5), a process which is catalysed by DNA-dependent RNA polymerase (RNAP), a nucleotidyl transferase enzyme (Floss and Yu, 2005; Mariani and Maffioli, 2009). In bacteria, the transcription process can be divided into a number of distinct steps in which the RNAP holoenzyme⁴ binds to duplex promoter DNA to form the RNAP-promoter complex, then a series of conformational changes leads to local unwinding of DNA to expose the transcription start site. RNAP

⁴ An apoenzyme is an enzyme which requires a co-factor but does not have it bound. A holoenzyme is the active form of an enzyme, consisting of the co-factor bound to the apoenzyme.

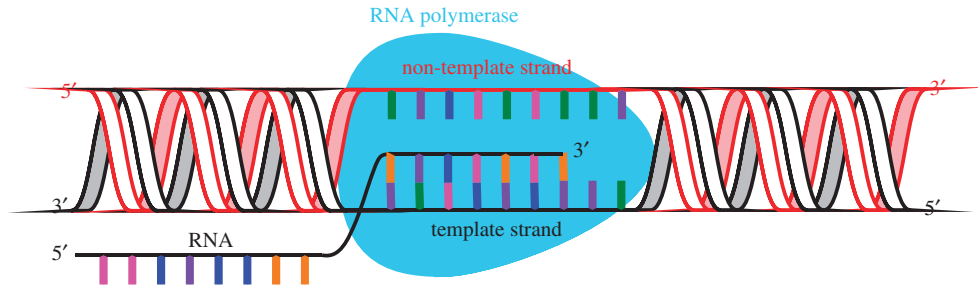


Figure 1.1.5 DNA transcription

can then initiate transcription, directing the synthesis of short RNA products, with synthesis of the RNA taking place in the $5' \rightarrow 3'$ direction (with the DNA template strand being read in the $3' \rightarrow 5'$ direction).

RNAP is a complex system, comprising five subunits ($\alpha_2\beta\beta'\omega$), each of which has a different function. The α subunits assemble the enzyme and bind regulatory factors, the β subunit contains the polymerase, the β' subunit binds non-specifically to DNA, and the ω subunit promotes the assembly of the subunits and constrains the β' unit. The core structure of RNAP is thought to resemble a crab's claw, with the active centre on the floor of the cleft between the two 'pincers', the β and β' subunits, and also contains a secondary channel, by which the nucleotide triphosphates access the active centre, and an RNA-exit channel (for a really good interactive tutorial showing the structure of RNAP, see <http://www.pingrysmartteam.com/RPo/RPo.htm>, last accessed 26 March 2012). Bacterial RNAP contains only these conserved subunits, while eukaryotic RNAP contains these and seven to nine other units (Ebright, 2000).

In bacteria, the transcription of a particular gene requires the binding of a further subunit, a σ factor (a transcription initiation factor), which increases the specificity of RNAP binding to a particular promoter region and is involved in promoter melting, and so results in the transcription of a particular DNA sequence. Once the assembly process is complete, the holoenzyme (the active form containing all the subunits: $\alpha_2\beta\beta'\omega\sigma$) catalyses the synthesis of RNA, which is complementary to the DNA sequence characterised by the σ factor (Figure 1.1.5) (eukaryotic RNAP also requires the binding of transcription factors, as do some bacterial RNAP). Proofreading of the transcription process is less effective than that involved in the copying of DNA, so this is the point in the transfer of genetic information which is most susceptible to errors. As we will see in Subsection 2.2.4, DNA-dependent RNA polymerase is the target of the rifamycin antibiotics.

Ochoa and Kornberg were awarded the Nobel Prize for Physiology or Medicine in 1959 'for their discovery of the mechanisms in the biological synthesis of ribonucleic acid and deoxyribonucleic acid' (http://nobelprize.org/nobel_prizes/medicine/laureates/1959/#, last accessed 26 March 2012).

Once the mRNA has been synthesised, it moves to the cytoplasm, where it binds to the ribosome, a giant ribonucleoprotein which catalyses protein synthesis from an mRNA template (**translation**). In 2009, Ramakrishnan, Steitz, and Yonath were awarded the Nobel Prize in Chemistry for their 'studies of the structure and function of the ribosome' (http://nobelprize.org/nobel_prizes/chemistry/laureates/2009/press.html, last accessed 26 March 2012).

The ribosome (Steitz, 2008), a large assembly consisting of RNA and proteins (ribonucleoproteins), has two subunits (30S and 50S in bacteria (complete ribosome 70S), 40S and 60S in eukaryotic cells (complete ribosome 80S)), and the large ribosome subunit has three binding sites, peptidyl-tRNA (P), aminoacyl-tRNA (A), and the exit (E) site, in the peptidyl transferase centre (PTC). Protein synthesis is initiated by the binding of a tRNA charged with methionine⁵ to its AUG codon on the mRNA. tRNAs (or charged

⁵In prokaryotes and mitochondria, this methionine is formylated (NH-CHO); in eukaryotic cytoplasm, it is free methionine.

tRNAs) then carry amino acids to the ribosome site where mRNA binds. tRNA has three nucleotides which code for a specific amino acid (a triplet) and bind to the complementary sequence on the mRNA. The ribosome moves along the mRNA from the 5'- to the 3'-end and, once the peptide bond has formed, the non-acylated tRNA leaves the P site and the peptide-tRNA moves from the A to the P site. A new tRNA-amino acid (as specified by the mRNA codon) then enters the A site and the peptide chain grows as amino acids are added, until a stop codon is reached, when it leaves the ribosome through the nascent protein exit tunnel (Figure 1.1.6). One thing which has probably already occurred to you is that every protein does not have a methionine residue at its amino terminus; this is a result of modifications once the protein has been synthesised. In bacteria, the formyl group is removed by peptide deformylase and the methionine is then removed by a methionine aminopeptidase. Although you might not agree, this is actually a simplification of protein synthesis, which also involves other processes and species, including initiation factors, elongation factors, and release factors.

In Section 4 we will look at several drug classes which target protein synthesis by interfering with different aspects of the ribosomal translation process highlighted above. As with DNA replication, these antibiotics target processes common to both prokaryotes and eukaryotes and so any selectivity will be based on subtle differences in the structures of the ribosomes in the different cell types.

1.1.3.4 Bacterial cell-wall synthesis

As mentioned earlier, bacteria have a cell wall and a cell (plasma) membrane, while mammalian eukaryotic cells only have a cell membrane. The prokaryotic cell wall is composed of peptidoglycan (a polymer consisting of sugar and peptide units) and other components, depending upon the type of bacterium.

Gram positive bacteria (which are stained dark purple/violet by Gentian violet-iodine complex) are surrounded by a plasma membrane and cell wall containing peptidoglycan (Figure 1.1.7) linked to lipoteichoic acids (which consist of an **acylglycerol** linked via a **carbohydrate (sugar)** to a poly(**glycerophosphate**) backbone, Figure 1.1.8).

The cell wall of **Gram negative bacteria** is more complex. They have a plasma membrane and a thinner cell wall (peptidoglycan and associated proteins) surrounded by an outer membrane of phospholipid and lipopolysaccharide and proteins called porins (Figure 1.1.9). The outer membrane is thus the feature of the Gram negative cell wall which represents the greatest difference to that of Gram positive bacteria. The **lipopolysaccharide (LPS)** consists of: a phospholipid containing glucosamine rather than glycerol (**lipid A**⁶), a **core polysaccharide** (often containing some rather unusual sugars), and an **O-antigen polysaccharide side chain** (Figure 1.1.10). As this outer membrane poses a significant barrier for the uptake of any non-hydrophobic molecules, the outer membrane contains porins: protein pores which allow hydrophilic molecules to diffuse through the membrane. As a result of their more complex cell wall and membranes, Gram negative bacteria are not stained dark blue/violet by the Gram stain, but can be visualised with a counterstain (usually the pink dye fuschin).

Finally, **mycobacteria** have a structure which includes a cell wall (Figure 1.1.11), composed of peptidoglycan and arabinogalactan, to which are anchored mycolic acids (long-chain α -alkyl-substituted β -hydroxyacids which can contain cyclopropyl or alkenyl groups, as well as a range of oxygenated functional groups); see Figure 1.1.12. Mycobacteria are resistant to antibacterial agents that target cell-wall synthesis (such as the β -lactams).

⁶ LPS is toxic and produces a strong immune response in the host. If Gram negative cell walls are broken by the immune system, the release of components of the cell wall containing the toxic lipid A results in fever and possibly septic shock.

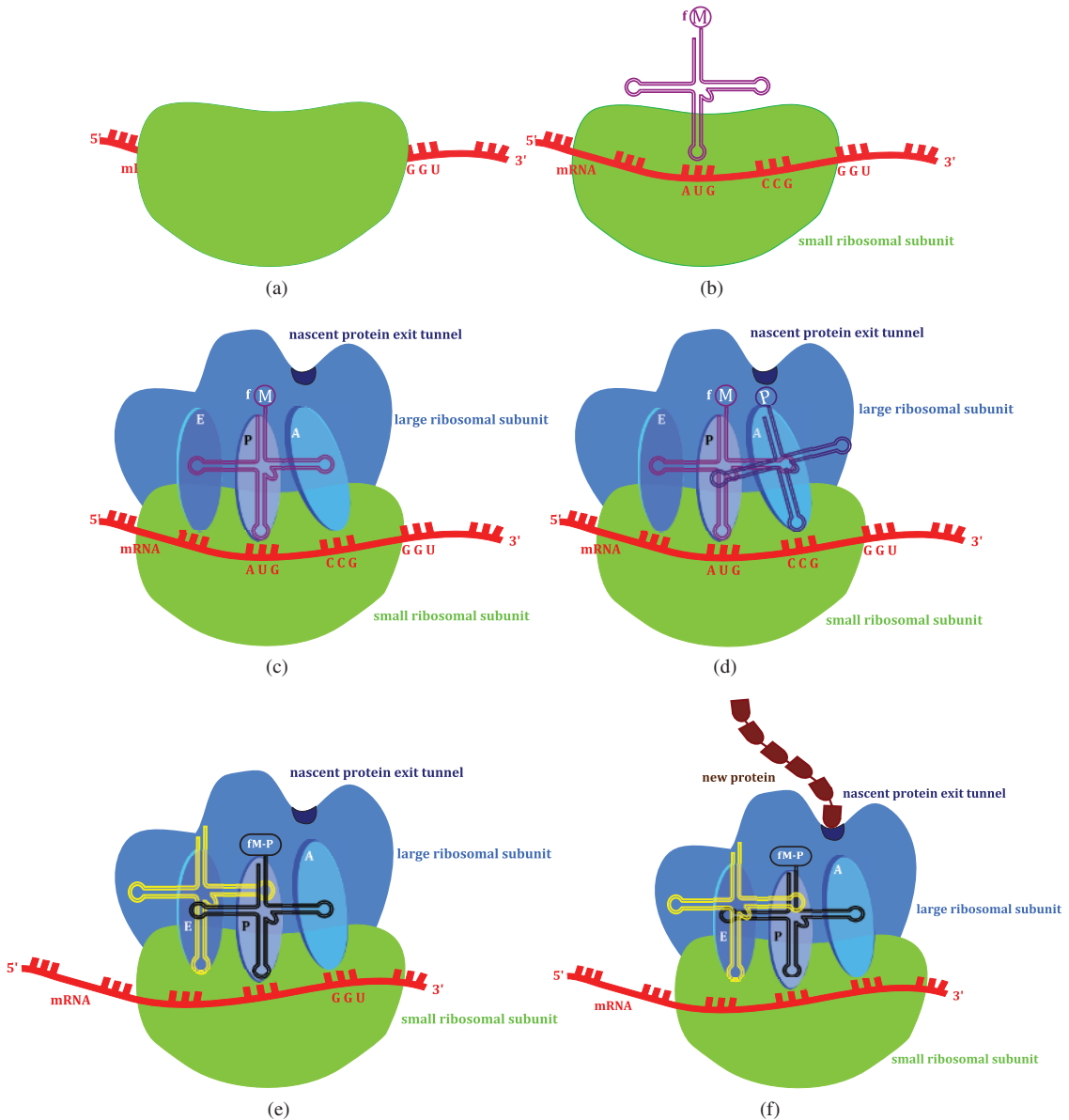


Figure 1.1.6 The sequence of events leading to protein synthesis on the ribosome: (a) the **small ribosomal subunit** binds to the **mRNA** product of transcription; (b) the initiation complex is formed as the **initiator tRNA (formyl-methionine)** binds; (c) the **large ribosomal subunit** binds – tRNA (formyl-methionine) bound in the P (peptidyl-tRNA) site of the peptidyl transfer centre (the small subunit is transparent to allow a view of molecular events within the ribosome); (d) the mRNA codon (CCG) dictates that **tRNA (proline)**, with an anticodon of GGC, binds to the A (aminoacyl-tRNA) site of the peptidyl transfer centre; (e) a peptide bond forms between methionine (M) and proline (P), the ribosome moves along mRNA in the 5' → 3' direction, **tRNA bearing M-P** binds to the P site, leaving the A site free to bind the tRNA encoded by the next three bases of the mRNA. The exit (E) site binds the **free tRNA** before it exits the ribosome; (f) as the amino acids are added, the **new protein** exits the ribosome into the cytoplasm via the nascent protein exit tunnel

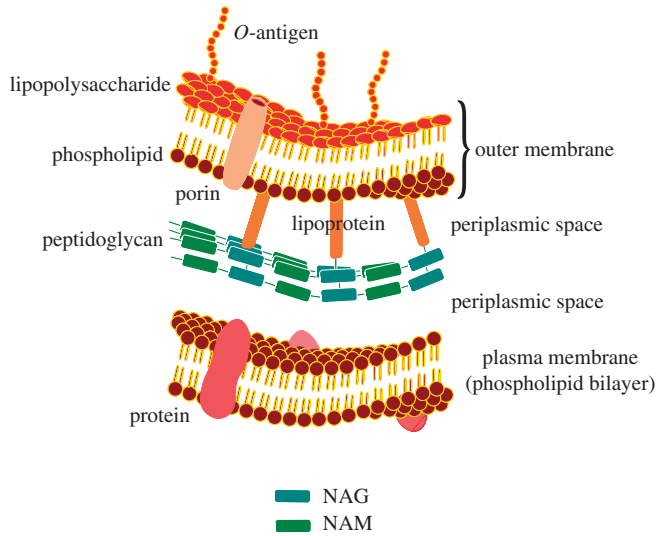


Figure 1.1.9 Schematic representation of the plasma membrane, cell wall, and outer membrane of Gram negative bacteria

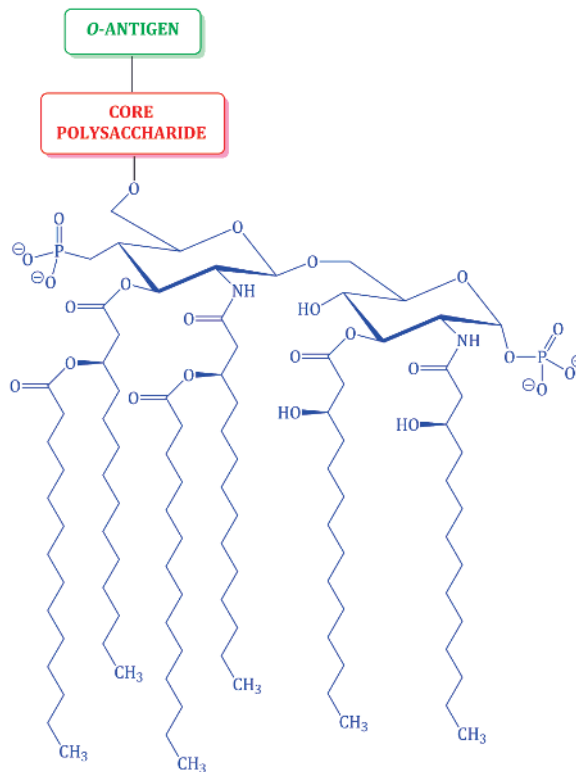


Figure 1.1.10 Schematic representation of the lipopolysaccharide from Gram negative bacteria

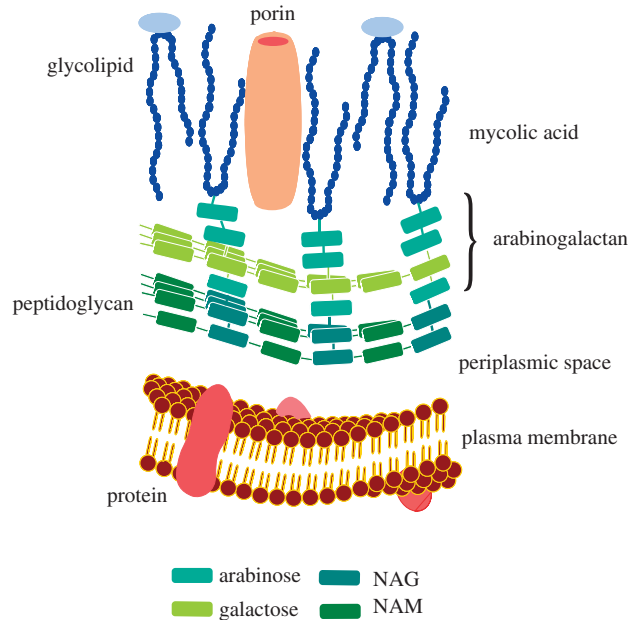


Figure 1.1.11 Schematic representation of the plasma membrane, cell wall, and mycomembrane (mycolic acid layer) of mycobacteria

Peptidoglycan (or murein) consists of parallel sugar backbones composed of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) (Figure 1.1.14). As with cellulose fibres, these chains have strength in only one direction and, in order to form the peptidoglycan structure which will give the cell wall its rigidity, they must be crosslinked. This crosslinking takes place via peptide chains attached to the *N*-acetylmuramic acid residue through the carboxylic acid group. These chains are then linked together in a series of steps catalysed by the penicillin binding proteins (PBPs), enzymes which are located at the outer portion of the plasma membrane and have a range of activities, including: *D*-alanine carboxypeptidase (removal of *D*-ala from the peptidoglycan precursor), peptidoglycan transpeptidase, and peptidoglycan endopeptidase.

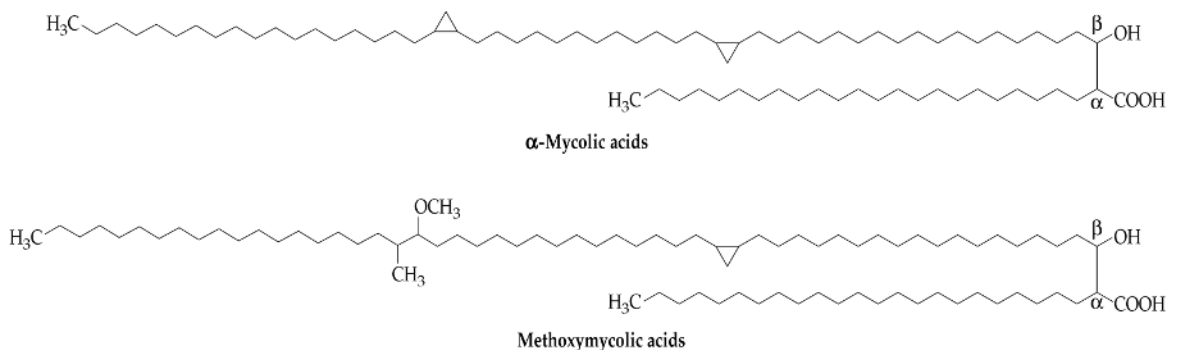


Figure 1.1.12 Examples of the structures of mycolic acids from mycobacterial cell walls (Langford et al., 2011)

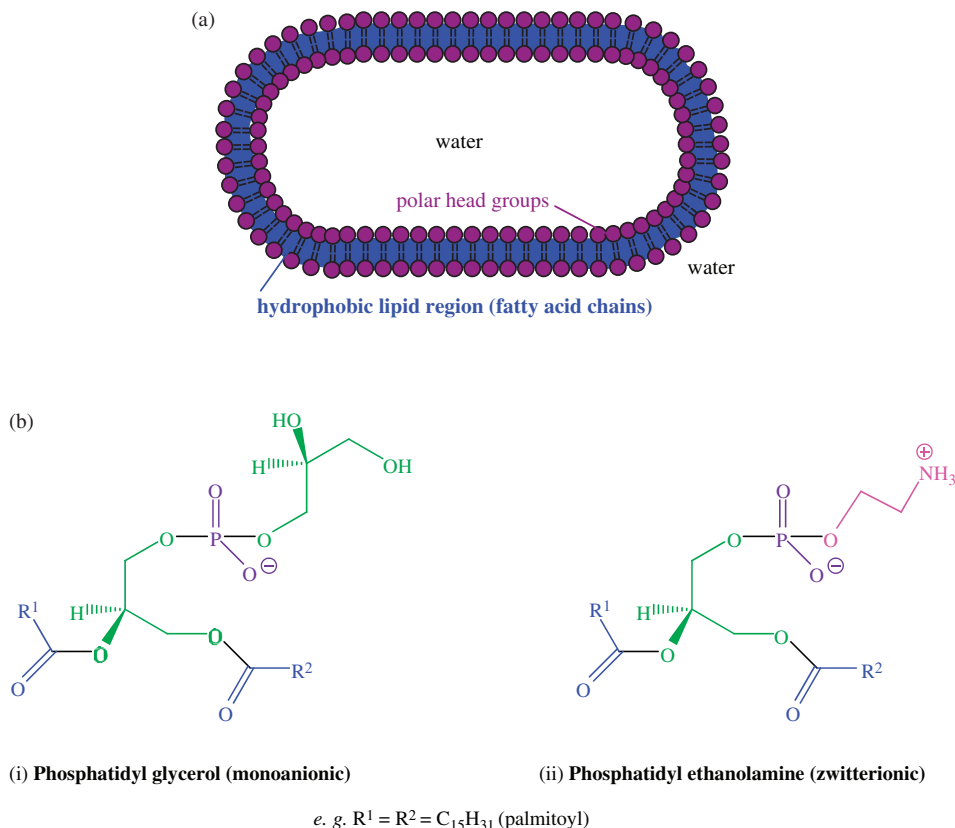


Figure 1.1.13 (a) Phospholipid bilayer formation, with the solvated polar head groups extending into the aqueous layer and the fatty acid chains forming the hydrophobic region; and (b) examples of (i) an anionic **phospholipid** derived from **fatty acids** and **glycerol** and (ii) a zwitterionic (doubly-charged) **phospholipid** derived from **fatty acids**, **glycerol**, and **ethanolamine**

As can be seen from Scheme 1.1.4, crosslinking of the peptide side chains involves the PBP acting as a serine-acyl transpeptidase (a serine residue at the active site attacks the terminal D-Ala-D-Ala sequence to generate an acyl-enzyme intermediate, with the loss of the terminal D-Ala⁷). Being an ester, this intermediate is more reactive than the amide it replaced and is attacked by the amino group of a glycine residue to give the amide crosslink.

As this crosslinking process is occurring at many places along the peptide-NAG-NAM chains, the net result is a rigid scaffold which gives the cell wall its strength.

As we've just seen, the formation of crosslinks in Gram positive bacteria involves the attack of the *N*-terminal amino of a glycine (Gly) residue on an acyl-enzyme intermediate to give a new Gly-D-Ala bond. The Gly residue is the last in a run of five Gly residues on a side chain of the pentapeptide attached to NAM. This sequence of Gly residues is attached to the pentapeptide through a dibasic amino acid (lysine, Lys). In

⁷The loss of this D-Ala from the carboxyl end of the peptide chain is catalysed by D-alanine carboxypeptidase activity of the PBP, a carboxypeptidase being the enzyme which removes the amino acid group from the carboxyl terminus (the end with the free COOH) of a peptide.

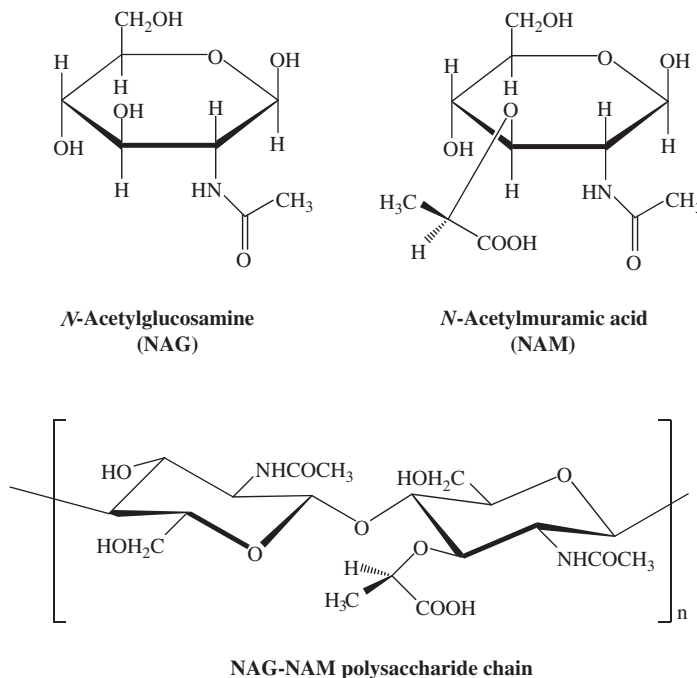


Figure 1.1.14 Structures of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) and a NAG-NAM polysaccharide chain

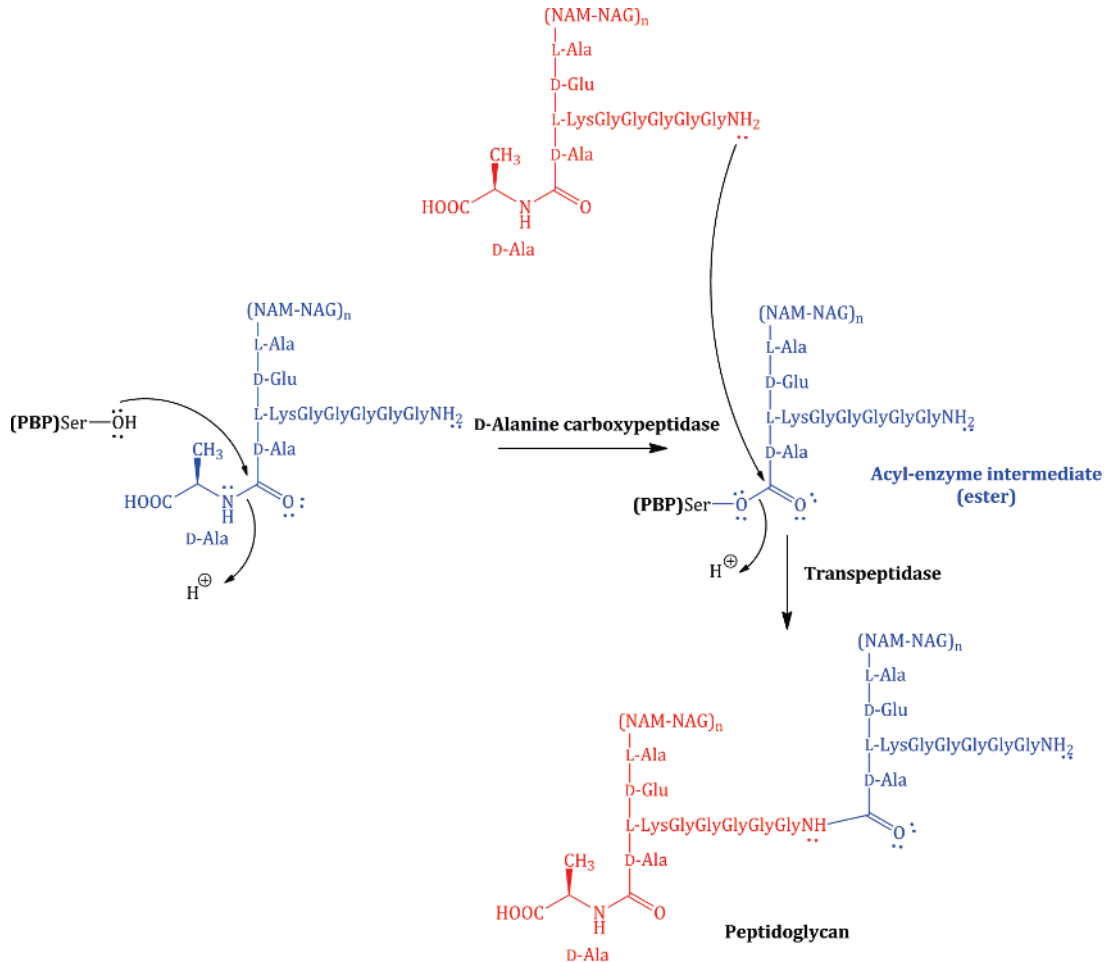
Gram negative bacteria, this dibasic acid is mostly diaminopimelic acid (A2pm) and the crosslink formation involves the direct attack of the ϵ -amino group on the acyl-enzyme intermediate, as shown in Scheme 1.1.5.

The biosynthesis of the cell-wall precursors takes place within the cytoplasm and these are then transported across the plasma membrane to the periplasmic space (van Heijenoort, 2001) and ultimately to the growing cell wall where they are required (Figure 1.1.15). The lipid which carries the ‘monomer’ units across the plasma membrane is derived from **pyrophosphoryl undecaprenol** and is shown in Figure 1.1.16. Once this lipid has delivered the monomeric unit to the growing cell wall, it returns to the cytoplasm to recruit another monomeric unit.

In Section 5, we will look at antibiotics which target cell-wall synthesis. As cell walls are unique to prokaryotic cells, these agents have the potential for selective toxicity: killing the bacterial cells but not affecting the eukaryotic (mammalian) cells. You are probably already aware that the β -lactams target cell-wall synthesis – the fact that the penicillin binding proteins (a rather misleading name as the function of these proteins is to catalyse peptidoglycan synthesis) are involved in cell-wall synthesis is a bit of a giveaway. Other agents which target the different steps involved in peptidoglycan synthesis are D-cycloserine and the glycopeptides, vancomycin and teicoplanin.

1.1.4 Bacterial detection and identification

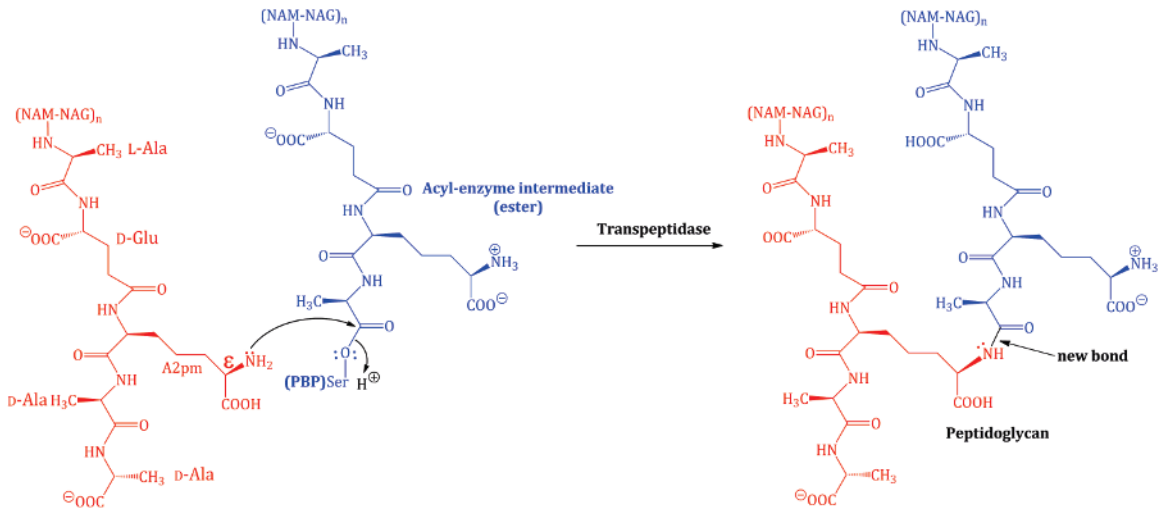
The detection and identification of bacteria is important in a variety of settings, including food hygiene, but we will concentrate here on the detection of pathogenic bacteria since, as we shall see, this is an increasingly important aspect of global health care systems.



Scheme 1.1.4 Penicillin binding protein (PBP)-catalysed peptidoglycan cross-coupling in Gram positive bacteria

In the UK, health care-associated infections (HAIs), including multiresistant organisms (MROs), were estimated to cost the National Health Service (NHS) in excess of £1 billion in 2004 (National Audit Office Report, 2004) and it has been estimated that the overall direct cost of HAI in the United States in 2007 was between \$36 and \$45 billion. If 20% of these infections are preventable then an annual health care saving of between \$5.7 and \$6.8 billion could be achieved in the United States alone (Scott, 2009). The development of new surveillance methods, which are key components of effective infection prevention and control, is, therefore, essential. Rapid identification of bacterial pathogens would also inform a more effective directed clinical treatment of the infection.

MROs are an increasing clinical problem, with particular concerns being cross-infection of patients and the transmission of resistance between these bacteria, which could ultimately lead to strains with limited, or no, susceptibility to current antibacterial agents. For example, although the incidence of glycopeptide-resistant enterococci (GRE) is currently much lower in the rest of the world than in the USA (where more than 20% of enterococcal isolates are vancomycin resistant), the report in 2003 of the *in vivo* transmission of vancomycin resistance from GRE to methicillin-resistant *Staphylococcus aureus* (MRSA) highlights the significant risk



Scheme 1.1.5 Synthesis of peptidoglycan crosslink in Gram negative bacteria

associated with having co-existing, non-isolated infections due to these pathogens (Chang *et al.*, 2003). It should also be noted that MRSA is susceptible to very few agents, including the glycopeptides (vancomycin and teicoplanin), quinupristin-dalfopristin, and linezolid, and that cases of meticillin- and quinupristin-dalfopristin-resistant *Staphylococcus aureus* have already been reported in Europe (Werner *et al.*, 2001).

Although there is no global consensus as to the most appropriate means of screening for MROs, timely active screening to identify colonised/infected patients should form the basis of an organism-specific approach to transmission-based precautions (NHMRC, 2010). Effective infection prevention relies upon rapid and reliable analysis of patient specimens and the introduction of contact precautions (such as patient isolation in a single-patient room or cohorting patients with the same strain of MRO in designated patient-care areas). For example, the use of a universal surveillance strategy was followed by a significant reduction in the rates of colonisation and infection of patients with MRSA (i.e. a change in the prevalence density from 8.91 to 3.88 per 10 000 patient days, compared to the case where no surveillance was undertaken) (Robicsek *et al.*, 2008).

From April 2009, the Care Quality Commission (www.cqc.org.uk) took over responsibility for health and social care regulation in the UK from the Healthcare Commission and ‘The Health and Social Care Act 2008, Code of Practice for the NHS on the prevention and control of healthcare associated infections and related guidance’, published in January 2009, describes how the CQC will assess compliance with the requirements regarding health care-associated infections, as set out in the Regulations made under Section 20(5) of this Act. Relevant NHS bodies must have, and adhere to, policies for the control of outbreaks and infections associated with both MRSA and *Clostridium difficile*, while acute NHS trusts must have similar policies for other specific alert organisms. With specific regard to MRSA, this policy should make provision for the screening of all patients on admission (including the screening of all elective admissions since March 2009 and the provision for screening of emergency admissions at presentation as soon as practical). This screening should then be used to inform the need for decontamination and/or isolation of colonised patients. Acute NHS trusts⁸ must also

⁸ A hospital is an acute NHS (or secondary care) trust.

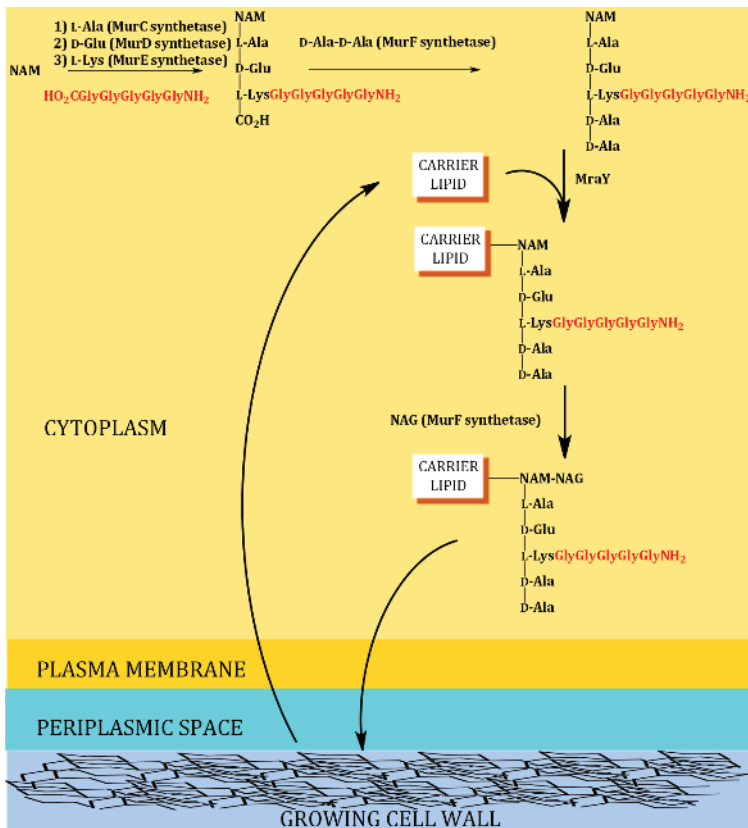


Figure 1.1.15 Assembly of the peptidoglycan precursors in Gram positive bacteria and transport to the growing cell wall. The enzymes which catalyse each step are shown in brackets (*MraY* = phospho-N-acetylmuramoyl-pentapeptide transferase). The GlyGlyGlyGlyGly sequence is shown in red only, to indicate that it is not entirely clear when in the cytoplasmic processes this pentapeptide is added to the lysine residue)

have policies for other specific alert organisms (for example, glycopeptide-resistant enterococci (GRE), *Acinetobacter* and other antibiotic-resistant bacteria, and tuberculosis (TB), including multidrug-resistant TB (MDR-TB)) (Health and Social Care Act, 2008; Groundwater *et al.*, 2009).

The Health Protection Agency publishes data derived from the mandatory surveillance of MRSA, *C. diff.*, and VRE bacteraemia, and the data show that January–March 2009 saw a 2.1% increase in MRSA bacteraemia compared to the previous quarter (but a reduction of 29% compared to the corresponding quarter in 2008),

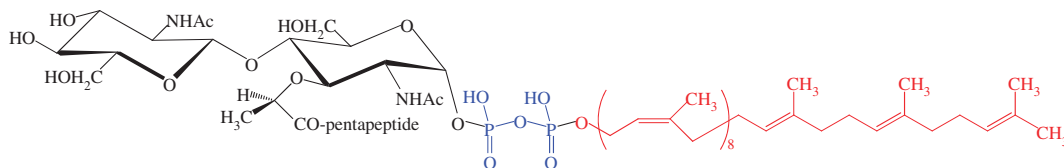


Figure 1.1.16 Lipid II, the immediate peptidoglycan precursor (Reprinted from J. van Heijenoort, *Nat. Prod. Rep.*, 18, 503–520, 2001, with permission of the RSC.)

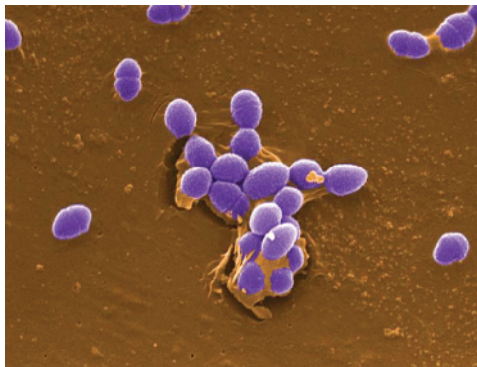
while there was a 34% decrease in the number of reported MRSA bacteraemias in the financial year 2008/2009 (HPA Mandatory Surveillance Report, 2008).

The need for rapid and simple methods for the detection of pathogenic bacteria, such as MRSA, GRE, NDM-1 metallo- β -lactamase producing organisms, *Pseudomonas aeruginosa*, Group B streptococci (*Streptococcus agalactiae*), and *Acinetobacter baumannii*, is hopefully self-evident.

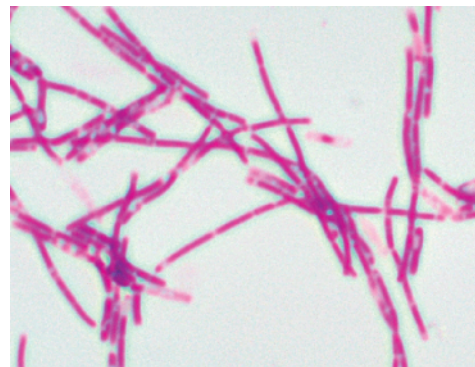
Traditionally, pathogenic bacteria are detected by Gram staining and microscopy and/or on the basis of their colonial appearance, after inoculation of a culture medium, which facilitates the growth of a wide range of organisms.

Prokaryotes have various shapes (Figure 1.1.17), and these, together with their appearance after the Gram stain, are used for their initial identification. The four basic shapes are:

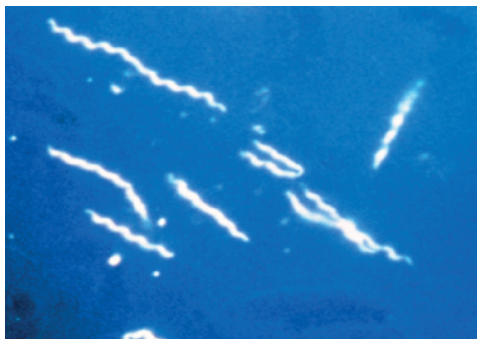
- Cocci (spherical);
- Bacilli (rod-shaped);
- Spirochaetes (spirals);
- Vibrio (comma-shaped).



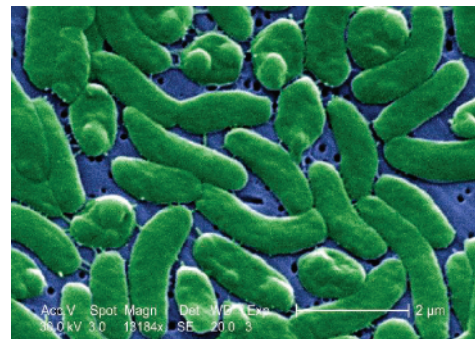
(a)



(b)



(c)



(d)

Figure 1.1.17 Examples of the different bacterial cell shapes: (a) Cocci (*Enterococcus faecalis* (photo ID12803)); (b) Bacilli (*Bacillus anthracis* (photo ID1064)); (c) Spirochaetes (*Borrelia burgdorferi* (ID6631)); (d) Vibrio (*Vibrio vulnificus* (ID7815)) (Image courtesy of Public Health Image Library, Images a) ID12803, b) ID1064, c) 6631 d) 7815, Online, [<http://phil.cdc.gov/phil/home.asp>, last accessed 29th March 2012].)

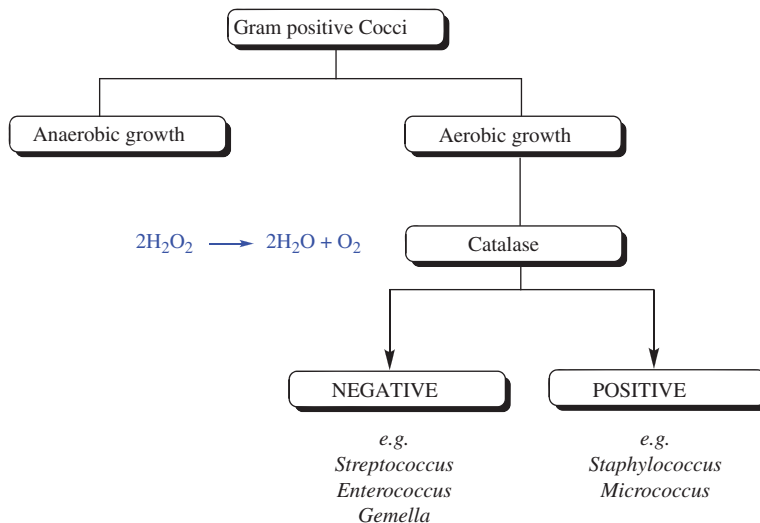


Figure 1.1.18 BSOP ID 1 Identification Flowchart for Gram positive Cocci (*catalase activity is detected via the production of oxygen upon addition of hydrogen peroxide*)

Bacterial identification requires the skills of an experienced clinical microbiologist and often requires further testing of commensal bacteria, which may have similar morphological characteristics to pathogenic bacteria. In the UK, bacteria are identified according to the National Standards (*Introduction to the Preliminary Identification of Medically Important Bacteria, BSOP ID 1*; <http://www.hpa-standardmethods.org.uk/documents/bsopid/pdf/bsopid1.pdf>, last accessed 30 April 2012) and a typical flowchart for bacterial identification is shown in Figure 1.1.18.

In the 1970s, the introduction of API strips, which consist of a series of miniaturized biochemical tests (such as the catalase activity mentioned in Figure 1.1.18), used in conjunction with extensive databases, allowed more rapid identification of bacteria and yeasts. There are now many API identification systems which can identify more than 600 bacterial species based on their reactivity in each of the biochemical tests.

Specific chromogenic media, in which a non-coloured enzyme substrate (a targeting molecule linked to a chromogenic compound) is added to the culture medium, have been employed for over 20 years in the detection of pathogenic bacteria (Figure 1.1.19) (Perry and Freydière, 2007). Ideally, this is a substrate for an enzyme which is unique to a particular bacterium, and cleavage of a key bond liberates a chromogen, which can be detected against a background of other, colourless colonies (as these do not contain the requisite enzyme for

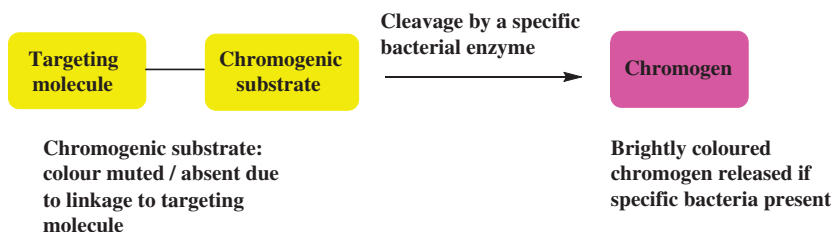


Figure 1.1.19 The principle behind the chromogenic detection of bacteria

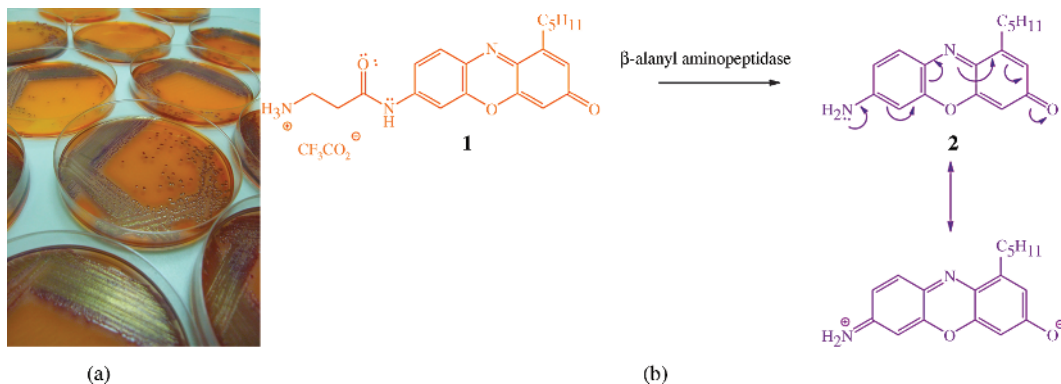


Figure 1.1.20 (a) Detection of *Pseudomonas aeruginosa* colonies using chromID (picture courtesy of Larissa Laine, Freeman Hospital, Newcastle upon Tyne, UK). (b) *P. aeruginosa* and the origin of the purple colour (Reprinted from A. V. Zaytsev, R. J. Anderson, A. Bedernjak, et al., *Org. Biomol. Chem.*, 8, 682–692, 2008, with permission of the RSC.)

cleavage of the chromogenic substrate). Often more than one chromogenic substrate can be employed in a single culture plate to help in the differentiation of commensal and pathogenic bacteria.

Among the benefits of the use of chromogenic media are that they can be sufficiently specific that no further testing is required, and that they can give indicative colours for bacterial colonies, although the time required (usually 24–48 hours) for the growth of the colonies (and so the development of colour) is a limiting factor in the development of a rapid test that could be applied to all patients on admission to hospital. Fluorogenic media (in which a fluorescent compound is released upon enzymatic cleavage) offer the possibility of more rapid bacterial detection and the simultaneous detection of more than one bacterium, if fluorogens with different emission wavelengths are used to target different enzymatic activities.

Chromogenic media have been employed for the detection of MRSA (Perry *et al.*, 2004), VRE (Randall *et al.*, 2009), ESBL-producing organisms (Ledebner *et al.*, 2007), and *C. diff.* (Perry *et al.*, 2010). A recent example of a medium that is selective for the detection of *P. aeruginosa*, the most common respiratory pathogen in patients with cystic fibrosis, employs the pale yellow-coloured β -alanyl-1-pentylresorufamine **1**, which is selectively cleaved by β -alanyl aminopeptidase (an enzyme specific to *P. aeruginosa*, *Burkholderia cenocepacia*, and *Serratia marcescens*) to give 1-pentylresorufamine **2**, which is retained within the bacteria and gives rise to purple colonies with a metallic sheen, which are easily detected by the naked eye (Figure 1.1.20) (Zaytsev *et al.*, 2008).

Molecular diagnostic methods (Tenover, 2007) offer the advantage that they are more rapid (results can typically be obtained within a few hours), can be highly specific, and, like the automated culture-based methods, can be performed in closed systems and have the capacity for automation. For example, one real-time multiplex PCR method⁹ for the detection of MRSA in a mixture of staphylococci employs DNA primers that are specific for the *mecA*- and *S. aureus*-specific *orfX* genes (to allow for variations in the staphylococcal cassette chromosome (SCC*mec*)), in addition to a series of molecular beacon probes for the detection of the single-stranded PCR product (Figure 1.1.21) (Huletsky *et al.*, 2004). Molecular beacons are single-stranded probes which have a specific DNA recognition sequence with a fluorescent dye at one end and a quencher at the other. If the recognition sequence matches the DNA sequence, the probe opens up and binds to the DNA, and

⁹ For an excellent description of PCR, see the Dolan DNA Learning Center's Web site (<http://www.youtube.com/DNALearningCenter#p/f/9/2KoLnIwoZKU>, last accessed 30 March 2012).

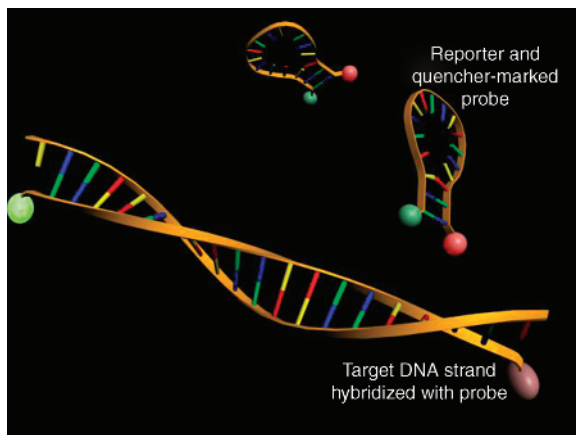


Figure 1.1.21 How a molecular beacon works (Reproduced from ‘Molecular Beacons’, Wikimedia, Online, [http://en.wikipedia.org/wiki/File:Molecular_Beacons.jpg].)

the resulting separation between the fluorescent dye and quencher means that the fluorescence is no longer quenched.

The SCC $_{mec}$ is a mobile genetic element that carries the *mecA*¹⁰ gene (which encodes the β -lactam-resistant penicillin binding protein PBP2['] (see Section 5)). In this study, of the 1657 MRSA isolates, 98.7% were detected in under 1 hour using this technique, with only 26 of the 569 methicillin-susceptible *S. aureus* (MSSA) strains being mistakenly identified as MRSA. Real-time PCR assays are available for the detection of MRSA (Cepheid Xpert MRSA (CA, USA) and BD GeneOhm MRSA (CA, USA)), based on the *mecA* gene, and of vancomycin-resistant enterococci (VRE) (BD GeneOhm VanR assay (CA, USA)), based upon the *VanA* gene. As we shall see later (in Section 5), the *VanA* phenotype produces a D-Ala-D-Lactate ligase which synthesises an ester (D-Ala-D-Lactate) rather than an amide (D-Ala-D-Ala) – this ester can still act as a substrate in peptidoglycan (cell wall) synthesis but has 1000-fold reduced binding affinity for vancomycin, which exerts its antibiotic effect by binding to the terminal D-Ala-D-sequence.

Another apparently appealing technique is the Matrix-Assisted Laser Desorption Ionisation-Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) detection of microorganisms (Hsieh *et al.*, 2008), but this has so far not been able to identify mixed organisms in the same blood culture specimen, requires a number of sample handling procedures, and, like PCR, requires expensive instrumentation and expert operators/analysts. In this technique, unlike most MS applications, the compounds giving rise to the individual MS peaks are not identified, but spectral fingerprints are obtained (which vary between microorganisms) (Figure 1.1.22). Among the compounds detected in the spectrum, some peaks (molecular masses) are specific to genus or species (and sometimes to subspecies). The mass spectra obtained are reproducible as long as the bacteria are grown and harvested under the same conditions, and protein extraction and analysis are also performed under standard conditions.

For example, a number of studies have attempted to differentiate between MRSA and MSSA based upon their MS profiles, which are different (with MRSA containing more peaks); however, no specific profile for MRSA has been identified (but individual strains do have very similar profiles) (Hsieh *et al.*, 2008).

¹⁰Don't worry about these genes and their functions just now. We'll discuss them more fully as they become important when discussing bacterial resistance to the different antibiotic drug classes.

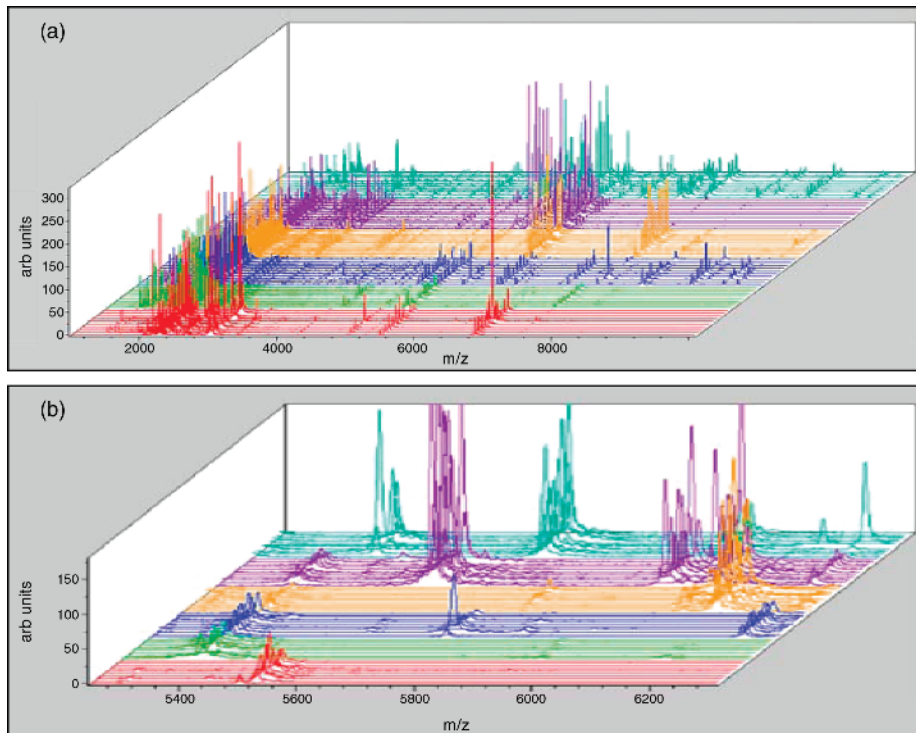


Figure 1.1.22 Examples of MALDI-TOF spectral fingerprints obtained from a number of micro-organisms: (a) full spectra obtained from strains of *S. aureus*, *Streptococcus group B*, *E. coli*, *Klebsiella pneumoniae*, *Salmonella serotype B*, and *Pseudomonas aeruginosa*; (b) expansion of the 5000–8500 Dalton/z region (Reprinted from S.-Y. Hsieh, C.-L. Tseng, Y. S. Lee, et al., *Mol. Cell. Proteomics*, 7, 448–456, 2008, with permission of The American Society for Biochemistry and Molecular Biology.)

1.1.5 Other than its mode of action, what factors determine the antibacterial activity of a drug?

In Subsection 1.1.2 we saw that the different structures of the bacterial cell walls in mycobacteria and Gram negative and Gram positive bacteria have an effect on the staining of these bacteria, and we might imagine that these structural differences would also have an effect on the uptake of antibacterial agents by the cells. This is undoubtedly the case, and infections due to Gram negative bacteria are often more difficult to treat than those caused by Gram positive bacteria. The antibacterial activity of a drug is not, however, solely governed by its mode of action and its ability to cross the cell membrane. There are a number of other factors which are important and which we will consider now. (For further information, see Adembri and Novelli, 2009; Barbour *et al.*, 2010.)

1.1.5.1 Bacteriostatic or bactericidal?

Agents which kill bacteria are referred to as **bactericidal**, while those which prevent them from multiplying (and thus rely on the host's immune system to kill and remove them) are **bacteriostatic**. Seems simple, doesn't it? In fact, some agents are bacteriostatic against some bacteria and bactericidal against others and whether an agent is bacteriostatic or bactericidal can even depend upon its concentration (see the aminoglycosides in

Section 4.1). **Bactericidal** agents are actually defined as those which produce a **'99.9% reduction in viable bacterial density in an 18–24 h period'** (Pankey and Sabath, 2004), so that agents which produce a 99.9% reduction after 48 hours, and are essentially bactericidal, are not classified as such. For example, the penicillins, vancomycin, and fluoroquinolones (which are generally referred to as bactericidal agents) kill *S. aureus* and *S. pneumoniae*, but are only considered to be bacteriostatic against enterococci *in vitro* as they do not produce a 99.9% reduction within 24 hours (Chambers, 2003).

1.1.5.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The efficacy of an antibiotic agent is usually indicated by the minimum inhibitory concentration (MIC), the lowest concentration which inhibits the visible growth of a particular organism after overnight incubation. The MIC is important for the following reasons:

- It gives an indication of the susceptibility of an organism to a particular antimicrobial agent: the lower the MIC, the more susceptible is the microorganism (this is particularly useful in the determination of bacterial resistance, as resistant strains will have higher MICs than those which are susceptible to an agent).
- It is used to inform the treatment of a bacterial infection (the antibacterial agent chosen would normally be that with the lowest MIC against the organism) and the dosing regimen for the antibacterial used.

MICs are determined by a standardised method (Andrews, 2001) and there are databases available which list the MICs for microorganisms (see for example the Antimicrobial Index Knowledgebase, <http://antibiotics.toku-e.com/>, last accessed 30 March 2012). For example, one of the methods of determining the MIC (which is used in the measurement of bacterial resistance) is the E-test, in which a strip containing an increasing concentration gradient of an antibacterial agent is placed on an agar plate inoculated with the bacterium of interest. After overnight incubation of the bacteria, a zone of inhibition appears as the bacteria grow around the strip and the MIC is determined as the position where this zone intersects with the strip.

Related to the MIC is the minimum bactericidal concentration (MBC), which is defined as the lowest concentration of an antimicrobial which will prevent the growth of an organism when subcultured on to antibiotic-free media.

1.1.5.3 Time- versus concentration-dependence

For bactericidal agents, the killing of bacteria is dependent upon both the concentration of the drug relative to the MIC and the length of time for which the bacteria are exposed to the drug. Bactericidal agents can exhibit either time- or concentration-dependent killing of bacteria, depending upon which effect predominates:

- If the bactericidal agent has time-dependent bactericidal action then, providing the drug concentration is above the MIC, the most important factor is the time the drug is in contact with the bacteria (the exact concentration doesn't matter) and there will be a constant rate of bacterial kill if the drug concentration is greater than the MIC.
- If the bactericidal agent has concentration-dependent bactericidal action then the absolute concentration of the drug is the most important factor. In this case, the rate of kill increases with increased drug concentration (which must be greater than the MIC) and one single large dose may be sufficient to eradicate all the pathogenic bacteria.

Some examples of concentration- and time-dependent antibacterial agents are given in Table 1.1.2 (Filho *et al.*, 2007).

Table 1.1.2 Concentration- and time-dependent antibacterial agents (Filho et al., 2007)

| Antibacterial agents | Bacterial killing |
|---|-------------------|
| <i>Aminoglycosides</i> (see Section 4.1, e.g. gentamicin) | Concentration |
| β -Lactams (see Section 5.1, e.g. ceftazidime) | Time |
| <i>Fluoroquinolones</i> (see Section 2.1, e.g. ciprofloxacin) | Concentration |
| <i>Macrolides</i> (see Section 4.2, e.g. azithromycin) | Time |
| <i>Oxazolidinones</i> (see Section 4.5, e.g. linezolid) | Time |

1.1.6 Bacterial resistance

It was Sir Alexander Fleming who first warned that the inappropriate use of penicillin could lead to the selection of resistant forms of *S. aureus*. He was proved correct as, in less than a year of the widespread introduction of penicillin, resistant *S. aureus* strains were discovered and there were epidemics of ‘hospital *Staphylococcus*’, a strain of *S. aureus* resistant to penicillin, chloramphenicol, erythromycin, and tetracyclines (Levy, 2002). Since the initial use of antibacterial agents, more and more bacteria have developed resistance, and some, such as *P. aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, have now developed such multidrug resistance that clinical isolates have emerged which are susceptible to only one class of antibacterial agent! (Falagas and Bliziotis, 2007).

Antibiotic resistance can be described as microbiological or clinical, with the latter being the failure to achieve an *in vivo* antibacterial concentration which inhibits the growth of the bacteria (Wickens and Wade, 2005).

Microbiological resistance can be classified as intrinsic or acquired. We will not concentrate much on intrinsic (or innate) resistance here, save to say that, if we think about it, it is obvious why some bacteria will have natural resistance to antibiotics. *Streptomyces* species, for example, produce a range of antibiotics, which we will study in the following sections, such as streptomycin, chloramphenicol, macrolides, tetracyclines. The *Streptomyces* species produce these agents to inhibit the growth of competing microorganisms and so must have natural resistance to these antibiotics themselves (otherwise they would be committing suicide by producing agents which kill themselves).

As we have said, resistance was observed soon after the introduction of antibiotics – the ability of resistant bacteria to transfer resistance to antibiotic-susceptible strains was linked to an ‘R factor’, later identified as a plasmid, which carries the genetic information required to confer the resistance, through a number of possible mechanisms:

- By alterations to a target enzyme or a group of bacterial enzymes linked to a biosynthetic pathway.
- By alterations to a protein, such as the PBP or the ribosome (a ribonucleoprotein).
- By alterations to the drug structure (rendering it inactive).
- By alterations to an efflux pump or porin, or other changes to the cell wall that confer impermeability.

We will come across many examples of each of these types of resistance in the sections on the individual classes of antibacterial agent.

Resistance to antibiotics is driven by three main conditions: pressure of selection (either continuously or periodically), the emergence of stable resistance genes, and the ability of these genes to be transferred via resistance vectors, such as plasmids, transposons, and epidemic strains (Amyes and Towner, 1990). Selective pressure is a result of *most* of the susceptible bacteria in the host being eliminated by the action of an antibacterial agent. Those bacteria which have resistance will be unaffected and continue to multiply, and the use of the antibacterial agent will thus lead to the selection of these resistant microorganisms.

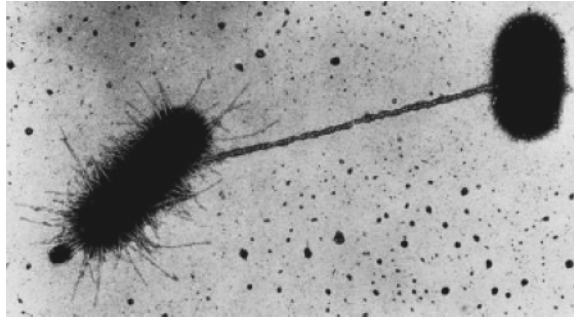


Figure 1.1.23 Bacterial conjugation (Reprinted from G. Karp, *Cell and Molecular Biology*, 2010, with permission of John Wiley & Sons.)

Resistance due to a chance mutation in the chromosomal DNA of the bacteria, leading to a beneficial change in a bacterial protein, can result in survival of that microorganism in the presence of an antibacterial agent. Although such chance mutations happen only once in every 10^7 cells produced, since some bacteria can divide every 30 minutes (or less), it may take as little as 6 hours for a mutant daughter cell to be produced. Once this altered (mutated) bacterial DNA has been produced, it can also be transferred to other bacteria by conjugation, transformation, or transduction (Alanis, 2005; Alekshun and Levy, 2007).

Conjugation is the most common mechanism for the transmission of resistance and is mediated by plasmids (circular fragments of DNA), which are transmitted between bacteria via a pilus – a hollow tube which is responsible for bringing the cells into intimate contact, thereby allowing the plasmids to transfer from one to another (Figure 1.1.23).

The most successful resistance plasmids are transferable between bacteria and carry the genes for resistance to a number of unrelated antibiotics; they can involve transposons¹¹ that can move from one plasmid to another, creating a mobile and effective communication of resistance information from antibiotic-resistant to antibiotic-susceptible bacteria, across strains, species, and even genera (Amyes and Towner, 1990). Although Gram positive and Gram negative bacterial resistance can have similar mechanisms, they are sufficiently different to be studied and reviewed independently. Gram positive bacterial resistance is most commonly plasmid-borne (Berger-Bächi, 2002; Woodford, 2005; Mlynarczyk *et al.*, 2010). Among pathogenic Gram negative bacteria, integrons¹² play a major role in the spread of antibiotic resistance genes. Integrons are predominantly found in plasmids, located in transposons, and are linked to the insertion, excision, and expression of mobile gene cassettes (White *et al.*, 2001).

Transformation involves the transfer of free DNA (in this case that containing the mutated sequence which confers some advantage on the recipient microorganism) between bacteria.

Transduction usually involves a virus known as a bacteriophage. Viruses require other cells to multiply and need to insert their genetic information into that of a host in order for it to be copied. In this case, the virus contains the mutated bacterial DNA and this becomes incorporated into that of the bacterium infected, so that every time this cell multiplies it will also produce the viral DNA (and so produce copies of the bacteriophage which can infect other bacterial cells).

¹¹ Transposons are DNA sequences that can move to new positions within the genome, using one of two processes with which you may, in your university life, already be familiar: ‘copy and paste’ and ‘cut and paste’.

¹² An integron is a genetic element that possesses a site at which further DNA, in the form of gene cassettes (usually linear sequences of a larger DNA molecule, such as a bacterial chromosome), can be integrated by site-specific recombination. It also encodes an enzyme – integrase – which mediates this site-specific recombination.

1.1.7 The ‘post-antibiotic age’?

As mentioned in the previous subsection, and as we will see throughout this textbook, bacteria have remarkable ability to develop resistance to the antibacterial agents with which they are treated. In addition, humans have been very complacent with the use of antibiotics, believing that there was a never-ending supply of antibacterial agents. We even continue to use them in animal feedstuffs as growth promoters (e.g. between 1992 and 1997, 56% of all antibiotics employed annually in Australia were used in feedstock). Avoparcin, a vancomycin analogue, was used as a growth promoter in pig feedstock, but was withdrawn from the market when a link was discovered between its use and the emergence of *Enterococcus faecium* expressing the *vanA* gene. The mass exposure of farm animals to antibiotics has probably been a key factor in the rapid emergence of resistance in some drug classes and the transfer of resistant bacteria to humans through the food chain (Turnidge, 2001).

As an example of the speed with which resistance can be transmitted around the world, we need only consider the metallo- β -lactamases (MBLs), a class of carbapenemases. As we shall see in Section 5.1, there are over 300 different β -lactamases, enzymes which cleave the β -lactam ring of these antibiotics, rendering them inactive. Different β -lactamases have different substrates and some, such as extended-spectrum β -lactamases (ESBLs), hydrolyse even third-generation cephalosporins (but not carbapenems) (Tumbarello *et al.*, 2004). MBLs, enzymes which hydrolyse the β -lactam ring of carbapenems through a mechanism involving zinc ion catalysis, have recently emerged in *Enterobacteriaceae* and represent the greatest threat to the use of these antibiotics. For example, the gene for the newest MBL, NDM-1 (*bla*_{NDM-1}), was only characterised in 2008 (it originated in India and Pakistan), but NDM-1 positive isolates have now been detected all across the globe, including the USA, UK, Europe, and Australia. These isolates have been detected in patients who have visited India (in an age where air travel can take us *and our infections* anywhere in the globe within 24 hours, this is not surprising), often for surgery (Walsh, 2010).

More worryingly, the plasmid which carries the NDM-1 gene also confers resistance to the macrolides, aminoglycosides, rifampicin, sulfamethoxazole, and aztreonam, leaving very few treatment options for infections caused by organisms such as *K. pneumoniae* or *A. baumannii* which possess it (Yong *et al.*, 2009).

The plasmid that carries the *bla*_{NDM-1} gene is just one example that confers resistance to a whole range of antibacterial agents, and as you will already be aware, there has been an alarming increase in the last decade in the number of bacteria which have resistance to more than one class of antibiotic, such as meticillin-resistant *Staphylococcus aureus* (MRSA). There are no prizes available for guessing one of the drugs which MRSA is resistant to, but the name doesn't indicate that MRSA strains are frequently also resistant to other clinically useful β -lactams, erythromycin (a macrolide) and ciprofloxacin (a quinolone), leaving very few agents available for treatment of MRSA infections (with vancomycin, a glycopeptide, being the agent of choice).

Other multiresistant bacterial infections include multidrug-resistant tuberculosis (MDR-TB, in which the tuberculosis is resistant to at least the two main first-line TB drugs – isoniazid and rifampicin) and extensively drug-resistant tuberculosis (XDR-TB, which is MDR-TB that is also resistant to three or more of the six classes of second-line drugs). Given that more than one third of the world's population has been exposed to TB, and that 90% of the exposed population has latent TB (asymptomatic), these are particularly worrying developments (World Health Organization, 2010).

We all have a vested interest in the availability of antibacterial agents for the treatment of infections (the authors have an even greater vested interest as, if there were no effective antibacterial agents remaining, there would be no need for students to study them, and hence no need for this book). These trends in increased levels of resistance and pan-resistant bacteria have been taken by some researchers to mean that we are entering (or have entered) a post-antibiotic era (Alanis, 2005; Walsh *et al.*, 2011).

Some of the questions which have to be asked in relation to the future availability of antibacterial treatments are:

- Will we soon witness the re-emergence of relatively simple life-threatening infections, which we believed were a thing of the past when clinicians had a range of potential antibacterial agents at their disposal?
- How soon will it be before some of the multidrug-resistant strains are no longer sensitive to any antibacterial agent (e.g. when vancomycin-resistant *Staphylococcus aureus* acquires the gene conferring resistance to all the other major classes of antibacterial agent)?
- What can we do to stop the further emergence of pan-resistant bacteria?
- What new classes of antibiotic are in development?

These are not easy questions to answer. Serious infections caused by multiresistant bacteria are already a global health care problem and antibiotic stewardship programmes are in operation throughout the world, through which hospitals seek to optimise antimicrobial prescribing in order to improve individual patient care and slow the spread of antimicrobial resistance (as well as reduce hospital costs) (MacDougall and Polk, 2005). Such programmes have a number of elements, including: restricting the dispensing of specific antibacterial agents to approved indications; the scheduled rotation of antibacterials used in a particular unit (if appropriate); further education of all members of the health care team to ensure the appropriate use of antibacterial agents (in particular the prescription of antibiotics with a narrower spectrum of activity as part of a directed clinical treatment regimen, rather than agents with a broad spectrum of activity in an empirical approach); and the education of the general public as to their expectations for the prescription of antibacterial agents and their use (e.g. advising patients to complete the course of antibiotic therapy, even though they may feel better after only a few days). The probability is that bacterial infections will soon emerge which have resistance to all the major classes of antibacterial agent, but, by adhering to such antibiotic stewardship principles, we may be able to delay this for a while yet.

You may well be thinking that all of the last couple of paragraphs are alarmist and that there will always be new members of a class of antibacterial agents (or even new classes) available to clinicians. Unfortunately, this is not the case. The ‘golden age’ of antibiotic discovery is now well gone and between 1983 and 2001 only 47 new antibiotics were approved in North America (by the US Federal Drug Administration (FDA) or Canadian Health Ministry). Of the nine new antibiotics approved since 1998, only two had a new mode of action and they are the only truly novel antibacterial agents launched in the last 30 years: linezolid (Pharmacia and Pfizer) and daptomycin (Cubist) (Overbye and Barrett, 2005).

And what of antibacterial agents in development? Surely every pharmaceutical company recognises the need for novel antibacterial agents and has research programmes aimed at discovering and developing them? Once again, the answers are not comforting. Pharmaceutical companies must make a profit to continue to be viable and so target chronic diseases, aiming for blockbuster drugs which will be given to patients for lengthy periods, in order to maximise their return on the massive costs associated with drug discovery and the short period during patent protection in which they, and they alone, can make a profit from the sale of a particular drug. Narrow-spectrum antibacterial agents, which will be used for the treatment of acute illnesses, and which are at risk of quickly becoming obsolete as a result of the emergence of bacterial resistance, are not commercially attractive to pharmaceutical companies. Lastly, as many antibacterial discovery programmes in industry were disbanded many years ago, much of the research expertise has been lost and would have to be re-introduced if companies were given incentives (such as increased patent lifetimes to allow them to maximise profits) to develop new discovery programmes.

All is not doom and gloom, however, and we will conclude the discussion of each class of antibacterial agent in the remainder of this book with a section on any new agents which are currently under development. New antibacterial targets obviously represent the ideal scenario for development, since resistance should develop

more slowly to an agent with a novel mode of action, but new agents in the already established classes, with different antibacterial spectra of activity, are welcome additions to the dwindling arsenal of agents which are available to clinicians.

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