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Culture

The bacterial spore: nature's survival package

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Introduction

Spores of *Bacillus* and *Clostridium* species are metabolically dormant and extremely resistant to acute environmental stresses such as heat, desiccation, UV and γ-radiation, mechanical disruption, enzymatic digestion and toxic chemicals. In addition to the spore's resistance to acute stress, spores can survive for extremely long periods in milder environmental conditions. Indeed, there are several reports suggesting that spores of *Bacillus* species can survive for millions of years in some special niches[1,2]. While this latter conclusion remains controversial, there is no doubt that spores of *Bacillus* and *Clostridium* species can survive for many, many years[3]. As a consequence of the persistence of spores and the ubiquity of spore formers in many different environments, spores are common contaminants of foodstuffs and if not dealt with appropriately in food processing may "return to life" via spore germination and outgrowth and then contribute to food spoilage and food poisoning[4]. In addition to food poisoning (*B. cereus, C. perfringens, C. botulinum*), there are a number of other human illnesses in which spores play a causative role including, wound infections (gas gangene: *C. perfringens*; tetanus: *C. tetani;* wound botulism: *C. botulinum*; intestinal infection: *C. difficile* and anthrax: *B. anthracis)*. Spores of *B. anthracis* persisting in soils are the common route whereby animals acquire pulmonary anthrax. The severity of this disease and the resistance of *B. anthracis* spores, particularly to desiccation, are undoubtedly major reasons that *B. anthracis* spores:

a) are considered a likely biological warfare agent; and
b) were used recently in terrorism incidents in the United States[5].

Spores and associated proteins of strains of a number of *Bacillus* species (*B. popillae, B. thuringiensis*) also cause lethal intoxications and infections of a variety of insect larvae.The spores of these species, or in some cases the toxins associated with the spores are currently used for insect control in agriculture. Spores of several *Bacillus* species are also currently used as probiotics for both humans and animals, and there is ongoing research into the use of:

a) *B. subtilis* spores as vaccine vehicles; and
b) spores of anaerobic *Clostridium* species in tumour therapy[6].

Sporulation

Spores of *Bacillus* and *Clostridium* species are formed in sporulation, a process triggered by starvation or environmental stress[4]. An early event in sporulation is generally an unequal cell division, generating a larger mother cell and a smaller prespore or forespore compartment. As sporulation continues, the forespore is engulfed by the mother cell, resulting in a "cell within a cell". The spore (also termed an endospore) then matures through a series of biochemical and morphological changes and eventually the mother cell lyses, releasing the spore into the environment. The whole process can take as little as eight hours in the laboratory, and may proceed at a high efficiency, with ≥75% of cells in a 24 hour culture having undergone sporulation.

Some strains of *B. subtilis* are naturally transformable with exogenous DNA and this has made genetic manipulation of these strains straightforward. This property and the determination of the *B. subtilis* genome sequence in 1997 have made this the organism of choice for detailed analyses of the regulation of sporulation (and also the mechanisms of spore resistance and spore germination). The sporulation "program" is driven by changes in gene expression in both time and space, as ~30% of *B. subtilis* genes change expression levels during sporulation with many groups of genes expressed only in sporulation:

a) at different times in the process; and
b) in the mother cell or the forespore.

Major mechanisms modulating gene expression during sporulation are the temporally and spatially specific synthesis and activation of proteins, termed sigma factors that associate with and determine the specificity of the cell's

IN THIS ISSUE

RNA polymerase, as these different sigma factors direct the RNA polymerase to recognise and transcribe new groups of genes. There is one new sigma factor that becomes active in the young sporulating cell prior to the unequal cell division, two that become active only in the mother cell, and two that become active only in the forespore. In addition, there are a number of sporulation-specific DNA binding proteins, both repressors and activators that further modulate sporulation gene expression in the sporulating cell compartments. There are also elegant mechanisms, often called checkpoints that co-ordinate gene expression in sporulation with major morphological milestones in the overall process. One example of such a checkpoint is the activation of a new sigma factor in the mother cell triggered by the engulfment of and proper gene expression in the forespore. These checkpoints presumably ensure that the differentiation of the mother cell and forespore remains "in register". While sporulation has been best studied by far in *B. subtilis*, analyses of sequenced genomes of other *Bacillus* and *Clostridium* species have indicated that major sporulation regulatory proteins of *B. subtilis* are generally conserved in these other species. Thus it is thought that the general mechanisms regulating spore formation are similar across these species.

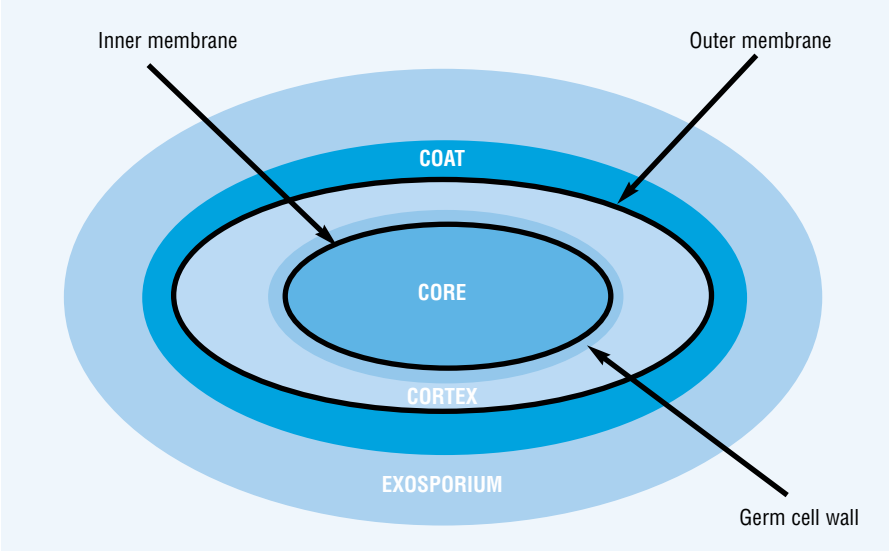

Figure 1. Spore structure (Note that all layers are not drawn to scale, and that the size of various layers varies significantly between spores of different species).

Spore structure

In addition to their metabolic dormancy and resistance, the spore has a very different structure from that of a growing cell, including several layers (see *Figures 1.* and *2.*) and many constituents that are unique to spores[7–9]. The outermost layer is the exosporium. While probably not present in spores of all species, in some spores the exosporium is by far the largest spore layer and is a loosely fitting, balloon-like structure containing carbohydrate and protein but mostly water. The exosporium contains a number of proteins and antigens unique to spores and for spores of *B. anthracis*, which have a very large exosporium, there is much work on such antigens for their potential utility in spore detection and generation of anthrax vaccines. However, the precise function of the exosporium in nature is not clear.

Underlying the exosporium is the spore coat composed largely of proteins. There are multiple proteins in the spore coat and often multiple coat layers and this structure and its constituents are again unique to spores. The coat protects the spore from destruction by lytic enzymes such as lysozyme and also many toxic chemicals.

The next layer in is the outer membrane and is very important in spore formation. However, in the dormant spore the importance of this membrane, even if it is a complete membrane, is not clear.

The next layer in is the cortex, composed of peptidoglycan (PG) that has a structure similar to that of growing cell PG but with several cortex-

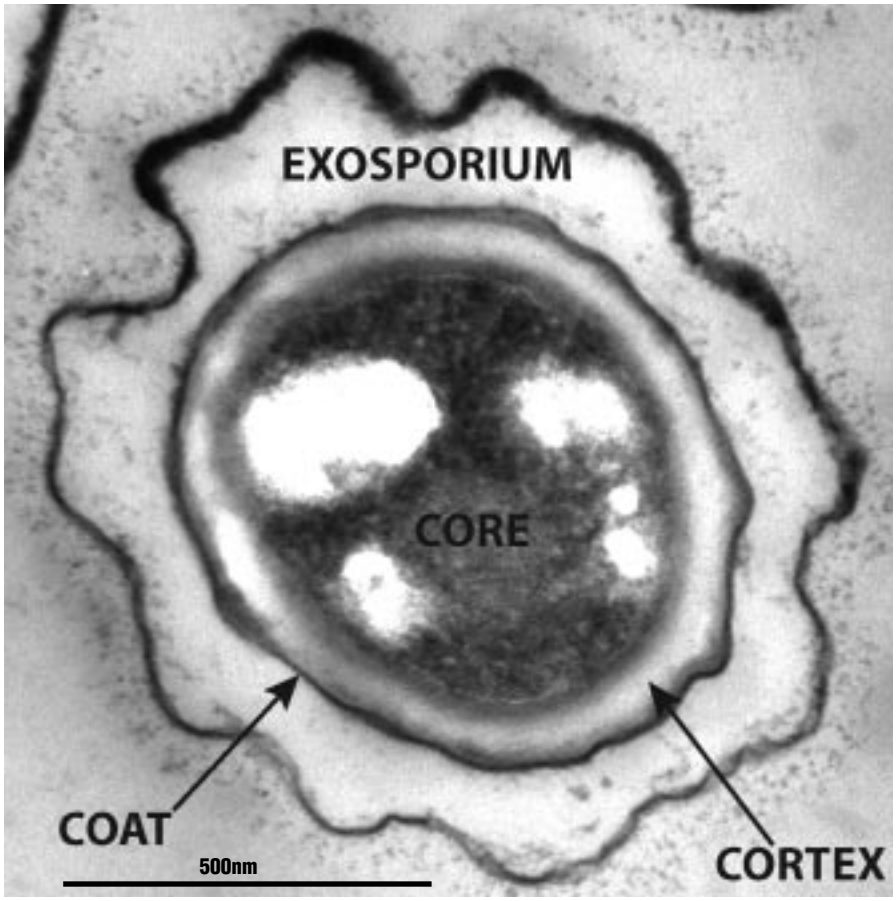

Figure 2. Electronmicrograph of a dormant spore of strain S69 *B. cereus*.

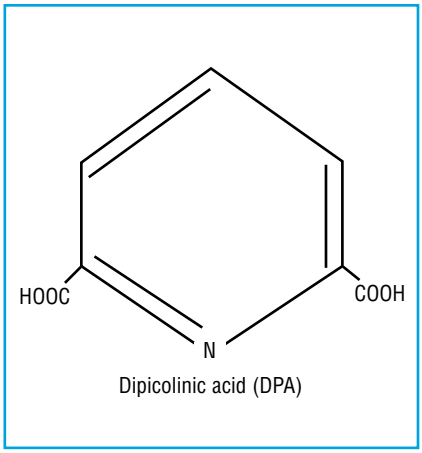

Figure 3. Dipicolinic acid structure

specific modifications. These cortex-specific modifications appear crucial for the recognition and eventual degradation of this layer when spores return to life in spore germination. Cortex formation during sporulation appears essential to establish both spore dormancy and much of the spore's resistance properties by causing a reduction in the core water content (see below), but how the cortex accomplishes this latter function is not known. There is a second PG layer under the cortex, termed the germ cell wall. The PG in this layer has a structure that appears identical to that of the growing cell PG and this layer becomes the cell wall of the spore when it returns to life.

The second spore membrane, the inner membrane, is next. This is a complete membrane but one with some novel properties. Despite a not unusual fatty acid and phospholipid composition, this membrane is relatively impermeable to small hydrophilic and hydrophobic molecules, and lipids in this membrane are immobile[10–12]. This membrane also appears to be very compressed in the dormant spore, as the inner membrane bounded volume increases ~2-fold early in spore germination without any membrane synthesis. The permeability of and lipid mobility in the spore's inner membrane also increase to values similar to those of the plasma membrane of growing cells upon completion of spore germination, again without any new membrane or macromolecular synthesis. While the reasons for the novel properties of the dormant spore's inner membrane are unclear, this membrane's low permeability contributes to the spore's resistance to some toxic chemicals.

Finally in the centre of the spore, termed the core, is the spore DNA and most spore enzymes and small molecules. Of particular note, with spores suspended in water, the core's wet weight as water is only 25–55% depending on the species, while in growing cells this value is ~80%[9]. The low core water content plays a key role in spore resistance to wet heat and in the spore's enzymatic dormancy. The spore core also contains an enormous amount (~20% of core dry weight) of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (*Figure 3.* Note that the carboxyl groups will be ionised at physiological pH.). This molecule is synthesised only in sporulation within the mother cell, is taken up by the forespore late in sporulation and is present exclusively in the spore core as a 1:1 chelate with divalent cations, predominantly Ca^{2+}. The core's high DPA level appears important in reducing spore core water levels and also in promoting and maintaining spore dormancy.

Spore dormancy

As noted above, the spore is metabolically dormant, and exhibits no significant (if any) metabolism of either exogenous or endogenous compounds. A striking feature of the spore's metabolic dormancy is the virtual absence of the common high energy compounds present at high levels in growing cells such as adenosine triphosphate (ATP), reduced pyridine nucleotides and acetyl-coenzymeA[13]. While spores are dormant, they do have significant pools of some metabolic substrates, in particular the glycolytic intermediate, 3-phosphoglyceric acid (3PGA), as well as the enzymes needed for metabolism of this and other substrates. However, these enzymes do not work on these subtrates in the dormant spore, and normally soluble proteins are immobile in the spore core, likely because of the core's low water content[13,14].

Table 1. Resistance of growing cells and dormant spores of *B. subtilis* to various treatments*

Treatment	Survivors – % Growing cell	Dormant spore
Wet Heat – 85°C; 30 min	$<10^{-4}$	79
Dry Heat – 90°C; 15 min	0.2	>90
Dry Heat – 120°C; 30 min	$<10^{-5}$	14
UV Radiation at 254nm – 315J/m^2	$<10^{-6}$	10
Freeze Drying and Rehydration – once	2	>90
Freeze Drying and Rehydration – 6 times	–	>90
0.5M HCl – 24°C; 30 min	$<10^{-6}$	65
4M H_2O_2 – 24°C; 30 min	$<10^{-6}$	70

* Data are taken from references 17 to 21.

Spore resistance

The other striking property of spores is their resistance[4,15]. For example, compared with growing cells, spores of *Bacillus* species in water are generally resistant to ~40° higher temperatures, can survive multiple cycles of freezing, drying and rehydration with no loss in viability, are 10- to 50-fold more resistant to UV radiation, are extremely resistant to acids, bases oxidising agents, aldehydes and alkylating agents, and can survive pressures as high as 8,000 atmospheres (*Table 1.*). Indeed, spores are so resistant and the potential danger so great if they survive processing/sterilisation (for example, if *C. botulinum* spores survive, there is the potential that the contaminated food can cause botulism), that food sterilisation procedures are designed with killing spores, in particular *C. botulinum* spores, and many tests of autoclave function use spore killing or spore enzyme inactivation as a readout.

Spore resistance is due to a variety of factors, including the spore coats, spore inner membrane impermeability, low core hydration, and specific mechanisms for protecting and repairing spore DNA[4,15,16]. The spore coats are important in protection against lytic enzyme attack on the spore cortex and also against killing by many, but not all chemicals, perhaps by functioning as "reactive armour". Loss of spore coats, either by mutation or chemical removal sensitises spores to lytic enzymes and to many chemicals. However, the spore coat plays little or no role in spore resistance to heat, desiccation or radiation. The low permeability of the spore's inner membrane appears important in protecting spores against chemicals such as formaldehyde and nitrous acid whose target is the DNA in the spore core[12]. Changes in inner membrane permeability generally result in parallel changes in spore resistance to nitrous acid and other chemicals that act in the spore core, although have no effect on resistance to chemicals that act outside the spore core. Of major significance in many spore resistance properties is the low water content of the spore core, and for spores of many species the lower the core water content, the higher the wet heat resistance. However, the mineralisation of the core with DPA and divalent cations also play a role in spore resistance to wet heat as does the identity of the divalent cation, with Ca^{2+} generally giving the most heat resistance.

Surprisingly, given the extremely high temperatures (≥100°C) at which spores of some species (eg. *Geobacillus stearothermophilus*) can survive for considerable times, there is little if any DNA damage associated with spore killing by wet heat. This indicates that spores must have some specific mechanisms for protecting their DNA from damage. Indeed, spore DNA is saturated with a group of small (60–75 residue), acid-soluble proteins (SASP) that protect the DNA against many types of damage[15,16]. The SASP are synthesised only in the forespore late in sporulation, are non-specific DNA binding proteins that are unique to spores and are degraded when spores return to life in spore germination. Binding of these proteins to DNA protects against damage caused by wet and dry heat, desiccation and many DNA damaging

chemicals including hydrogen peroxide, formaldehyde and nitrous acid. SASP binding also causes significant changes in DNA structure, with an accompanying dramatic change in the DNA's UV photochemistry[15,16]. This change in DNA photochemistry is a major factor in the spore's resistance to UV light. The other factor responsible for minimising spore DNA damage is DNA repair, since many DNA repair enzymes and pathways can operate when spores germinate and return to life, and at least one of these repair mechanisms is unique to spores[15,16].

Spore germination

While spores can remain dormant for extremely long periods, they are continually sensing their environment for the presence of nutrients using a group of receptors located in the spore's inner membrane[22]. Different receptors respond to different nutrients and at least some receptors appear to act cooperatively in sensing mixtures of nutrients. Nutrients to which these receptors respond include amino acids, sugars and purine nucleosides. In some fashion, the binding of these nutrients to their receptors triggers the initial events in germination, including the release of DPA and monovalent and divalent cations from the spore core and the parallel influx of water[22]. This process, which has been termed Stage I, may take ≤1 minute for an individual spore, although longer in a population of spores, in which individuals generally exhibit varying lag periods between addition of nutrients and the initiation of germination. Spore germination can also be triggered by exogenous Ca^{2+}–DPA, cationic surfactants such as dodecylamine or very high pressures (1,000–8,000 atmospheres)[22]. This latter mechanism for triggering of spore germination has drawn considerable interest form the food industry, as high pressure processing holds out the possibility of reducing spore burdens in foods with minimal reduction in food quality, since germinated spores have lost the high resistance properties of the dormant spores[23].

Metabolism of neither exogenous nor endogenous nutrients is required for events in State I of spore germination; even after these events are completed the spore still does not contain ATP, core enzymes still do not work and the core water content remains well below that of a growing cell[22]. However, the rise in core water in Stage I of germination does result in a significant decrease in spore wet heat resistance.

The germination process now continues into Stage II through the stimulation of hydrolysis and eventual degradation of the spore cortex, a spore layer that appears to act as a strait jacket restricting the swelling and full rehydration of the spore core. Again, the completion of Stage II in the germination process takes only minutes for individual spores and does not require metabolism of exogenous or endogenous subtrates. Action of the cortex-lytic enzymes in Stage II is triggered by events in Stage I of germination, in particular the release of DPA and its accompanying divalent cations. Interestingly, spores have multiple and redundant cortex-lytic enzymes whose activity is triggered by different signals. Presumably the presence of redundant cortex-lytic enzymes in spores is a fail-safe mechanism in case one enzyme is lost or not activated. The degradation of the cortex by these enzymes then allows the core to expand and take up more water until the core hydration level reaches that of a growing cell, thus completing spore germination.

Spore outgrowth

The fully germinated spore then begins outgrowth, as the increase in core water content in Stage II of germination allows initiation of enzyme action within the core, resulting in SASP degradation to amino acids and metabolism of stored energy reserves such as 3PGA and the amino acids generated from SASP degradation, as well as metabolism of exogenous compounds[22]. This metabolism generates nucleoside triphosphates including ATP, as well as other common high-energy compounds. Since SASP degradation early in outgrowth frees the DNA from the coating of SASP, mRNA synthesis also beings followed by protein synthesis. At this point the outgrowing spore is now well on its way to becoming a vegetative cell, and with the replication of its DNA, an event that may take place as soon as ~45 min after initiating germination, the spore's long journey is complete, having become a growing cell once again.

Acknowledgements

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The use of chromogenic enzyme substrates in microbial identification

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Introduction

Bacteria lack suitably diagnostic morphological characteristics so identification has largely relied on their ability to degrade substrates or produce certain biochemical substances as end-points of metabolism. Although molecular biology aids identification, it is still necessary to use biochemical tests to screen isolates from the vast number of food and clinical samples examined every year.

Early biochemical tests were often developed and used empirically and it was only much later that their underlying biochemical basis became apparent. More recently, microbiologists have focused on the hydrolase group of enzymes because of their greater discriminatory properties. This is due to the ready availability of substrates; for example, *Escherichia coli* can easily be detected in the presence of other organisms in water samples by its ability to cleave β-glucuronide substrates. In addition, hydrolases lend themselves to assay procedures that give rapid results.

Many hydrolase enzymes have a fairly strict requirement for the structure of the component on one side of the bond to be hydrolysed, but a low requirement for the group on the other side. Thus, glycosidases exhibit specificity not only for the sugar type but also for its steric conformation and for the conformation of the glycosidic bond. On the other side of the bond, the group, which in natural substrates usually yields an alcohol, is of much less importance and may be anything from another sugar to a phenol. This is useful in that a chromogen can be attached and specificity for a glycosidase is retained while activity of the enzyme may easily be monitored by observing colour production. Examples of hydrolase substrates that are available for diagnostic purposes are shown in *Table 1*.

Chromophores used for the detection of hydrolase activity

Nitrophenol

Some of the earliest artificial enzyme substrates produced were based on nitrophenol, a compound which at a pH above the pKa forms the yellow nitrophenoxide ion. If electron-attracting groups are attached to the oxygen atom of nitrophenol it cannot donate electrons and the compound is colourless. Hydrolase reactions may be monitored by observing the amount of yellow colour produced (*Figure 1*). Other carboxylic or inorganic acids can be linked to the oxygen to form substrates for longer chain esterases, phosphatases or sulphatase. In addition, saccharides can be attached to form glycosides.

o-Nitrophenol is cheap and the synthesis of substrates from it is fairly uncomplicated. This has led to its widespread use but it does suffer from two notable drawbacks: the extinction coefficient for the coloured form is fairly low and it is, therefore, not particularly sensitive. Also, it is fairly water soluble. The second characteristic renders it unsuitable for use in cell staining or in agar plates but it is still much used in liquid tube assays. Indeed the ONPG (Ortho-nitrophenol-β-D-galactoside) test still forms an important part of many bacterial classification tables.

Phenolphthalein

Phenolphthalein diphosphate[1] is hydrolyzed by phosphatase to phenolphthalein which is determined in alkaline conditions at 530 nm. Both phosphate groups must be split off before colour can be formed and phenolphthalein monophosphate has, therefore, been described as a better substrate[2]. Similarly, Coleman[3] used thymolphthalein monophosphate as a substrate.

Indoxyl

Indoxyl is a colourless, water soluble compound that is rapidly air-oxidised to the intensely coloured, insoluble, dimeric compound, indigo blue. Indoxyl is stabilised by the formation of esters or glycosides and hydrolase activity may be monitored by the production of indigo blue (*Figure 2*). Indigo blue is highly insoluble and when indoxyl substrates are used in agar plates, colouration is restricted to the cellular mass or to the agar immediately adjacent to the microbial colony. This enables colonies of a species containing the relevant hydrolase enzyme to be readily recognised in a mixed culture.

Substitution in the aromatic ring of indoxyl with halogens or attachment of a methyl group to the heterocyclic nitrogen produces pronounced shifts in the wavelength of absorption of the dimeric precipitate (*Table 2*).

Table 1. Examples of commmercially available hydrolase substrates.

Enzyme	Chromogenic substrates
Aminopeptidase	A multitude of substrates are available, containing single amino acids rising to quite long peptide lengths.
Esterase (carboxylic)	A range of substrates containing various fatty acid chain lengths: C2, C4, C6, C8, C9, C10, C12, C14, C15, C16 and C18.
Esterase (inorganic)	Phosphate, phosphodiester, venom phosphodiester, sulphate.
Glycosidase	α-L-Arabinoside, β-D-Cellobioside, α and β-L-Fucoside, β-D-Fucoside, α- and β-Galactosaminide, α- and β-D-Galactoside, α- and β-D-Glucosaminide, α- and β-D-Glucoside, β-D-Glucuronide, β-Lactoside, α- and ≤-D-Maltoside, α- and β-Mannoside, α-L-Rhamnoside, β-Xyloside.
Others	Substrates for lysozyme and phosphatidylinositol phospholipase C.

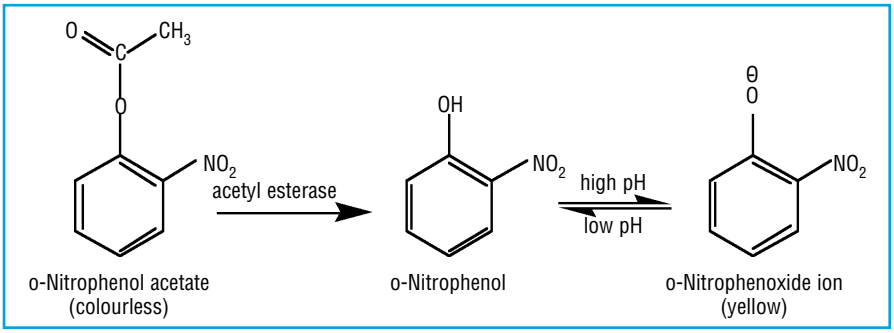

Figure 1. Production of the yellow nitrophenoxide ion from o-Nitrophenol acetate.

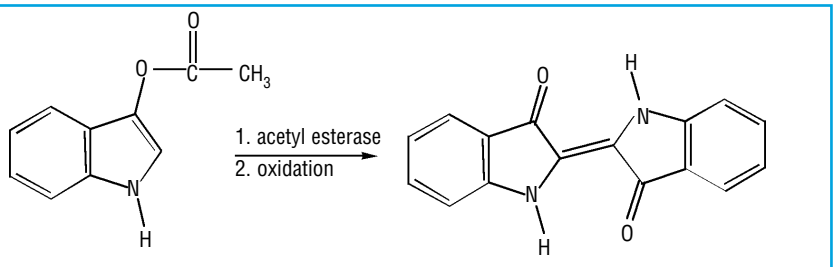

Figure 2. Indigo blue produced by enzyme treatment and oxidation.

Other chromogens

The production of colour from indoxyl substrates is dependent on oxidation. This presents problems if agar plates are used to detect organisms in anaerobic conditions. A β-galactosidase substrate based on p-naphthol-benzene has been described[4] which overcomes this problem and gives highly restricted zones of colour around colonies.

Indoxyl esters are very poorly water soluble and it is often difficult to produce agar plates containing these substrates without a precipitate forming. Esters based on 4 - [2 - (4 - octanoyloxy - 3,5 - dimethoxyphenyl) - vinyl] - quinolinium - 1 - (propan - 3 - yl carboxylic acid) (SLPA) are soluble and produce a burgundy-red colour around colonies[5].

Metal chelates

Another approach to colour production from enzyme substrates is to use a compound that is capable of forming a chelate with metals present in the test medium. One of the first to be used was the natural β-glycoside, esculin. This is broken down by β-glucosidase and the esculetin that is released complexes with ferric ions in the medium to form a black, poorly water soluble, compound (*Figure 3*). This has been used in both liquid and solid agar plate media. However, in the latter there is considerable spreading of colour. A much more hydrophobic variation of the esculetin structure, cyclohexenoesculetin, has been synthesized which produces much reduced colour spread[6].

8-Hydroxyquinoline-β-D-glucoside and β-D-glucuronide have also been used in enzyme assays[7]. 8-Hydroxyquinoline is released on hydrolysis and forms a dense black, insoluble chelate with ferric ions in the assay medium. However, this is toxic, particularly for Gram-positive organisms and it is rarely used microbiologically. A much better candidate for use in agar plate bacterial tests is alizarin. This is non-toxic and forms brightly coloured complexes with metals, the colour of which is dependent on the metal used[8]. The colouration is highly localised and it is described by the authors as a highly sensitive reagent.

Table 2. Effect of substituents on the colour of indigo compounds

Derivative	Colour of precipitate	Name	Absorption (nm)
None	blue	Y™	680
N-methyl	green	Green™	665
5-bromo, 4-chloro	blue	X -	615
5-iodo	purple	iodo	575
5-bromo, 6-chloro	magenta	Magenta™	565
6-chloro	salmon	Salmon™	540

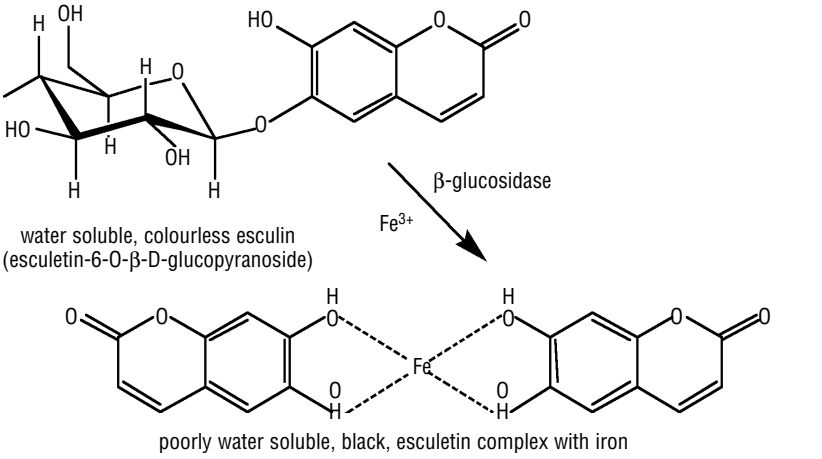

Figure 3. Hydrolysis of esculin to form a black esculetin/iron complex.

Nitroaniline

Peptidase enzymes are usually detected using the chromogen p-nitroaniline. This behaves in a similar manner to nitrophenol in that attachment of an electron withdrawing moiety, such as an amino acid or peptide, to the amine group renders the compound colourless. Hydrolysis of the peptide bond releases free nitroaniline which is yellow (*Figure 4*).

One of the most useful aminopeptidase enzymes for bacterial identification is L-alanine aminopeptidase. Significant enzyme activity is restricted almost entirely to Gram-negative micro-organisms. Although some Gram-negative organisms do not possess this enzyme, most notably *Campylobacter*, all Gram-positive or Gram-variable micro-organisms examined do not display activity or give a very weak reaction[9,10]. The aminopeptidase test thus provides a reliable method for distinguishing Gram+ and Gram– micro-organisms.

p-Nitroaniline suffers from the same drawbacks as nitrophenol in that it has a low extinction coefficient and it is water soluble. However, a poorly water soluble chromogenic alternative for use in agar plates is not commercially available. If sensitivity is a requirement, it, or any other suitable amine containing molecule, may be derived enzymatically. Thus, non-coloured β-naphthylamine released from a peptide substrate may be tested with a dimethylaminocinnamaldehyde reagent. Within a few minutes of addition, a red colouration is produced as the Schiff base is formed. This test can be adapted to use on cards or paper strips. A colony from an agar plate is rubbed onto a card containing the substrate, buffer is added, and the card incubated for 10 to 20 minutes. The reagent is then added and if the organism contains the relevant enzyme a red colour will appear.

Uptake of Chromogenic substrates

Lipophilic chromogenic substrates, such as esters, can enter the bacterial cell by passive diffusion. This occurs in Gram-positive but not Gram-negative organisms unless the outer cell membrane has been made permeable by detergents or chelating agents. Thus, bile salts are often added to *Salmonella* selective media

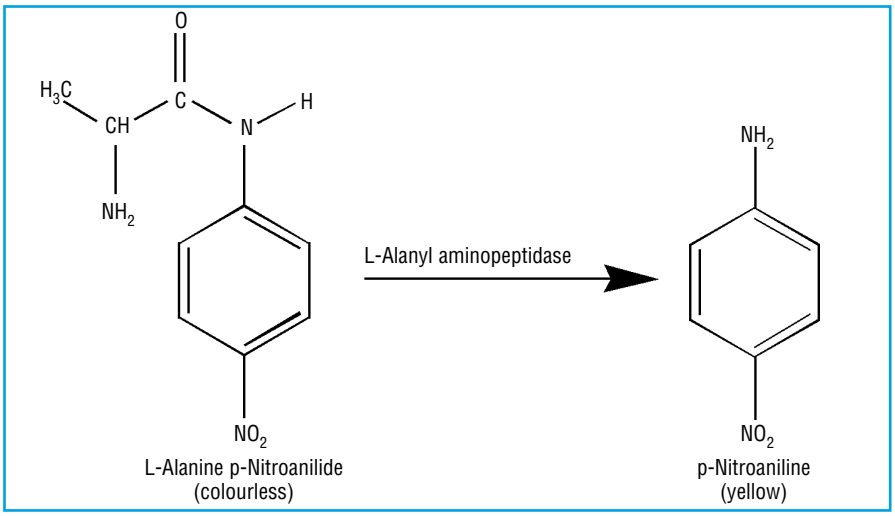

Figure 4. Production of yellow p-Nitroaniline from L-Alanine-p-nitroaniline.

not only to inhibit Gram-positive organisms but to permit entry into the cell of the chromogenic marker indoxyl caprylate.

Active transport involves accumulation of the substrate against the osmotic gradient in the cell at concentrations far in excess of that found in the medium. There are two main methods of active transport of glycosides in bacteria. The symport system uses an ion gradient to drive uptake of the substrate. It has a broad specificity but a low sensitivity and higher concentrations of a sugar are required for growth. A limitation of this system is that it is not active in stressed or stationary phase cells.

The other main active transport system is the more complex phosphoenol-pyruvate: phosphotransferase system (PTS)[11]. This does not rely on an ion gradient and is functional at every stage of growth of the organism. The PTS system generally has a narrower specificity but has a greater sensitivity than symport transport and has a greater ability to concentrate a substrate relative to a concentration gradient. This type of permease, however, is more sensitive to the structure of the aglycone and this has limited the use of some chromogenic substrates.

The highly sensitive but discriminatory PTS system is useful when substrates are at low concentration, and the more general symport system is used when substrates are available at high concentration.

Peptide uptake is again active and is by one of three systems depending on the peptide structure: dipeptide-, tripeptide- or oligopeptide-permease. The permease pathway is the major route of entry for most chromogenic peptidase substrates.

The ability of an organism to cleave a chromogenic substrate is dependent on possession of both a hydrolase enzyme and a functional permease system. A further complication encountered when using glycosidase substrates is that glycosidases are often inducible. This is particularly true of β-galactosidase which is strictly controlled by the *lac* operon. A natural substrate, such as lactose, may be used to induce the enzyme but activity may then be reduced by competitive inhibition. This is overcome by the addition of the inducer, isopropyl-β-D-thiogalactopyranoside (IPTG), which is 10,000 times more inductive for the *lac* operon than lactose itself.

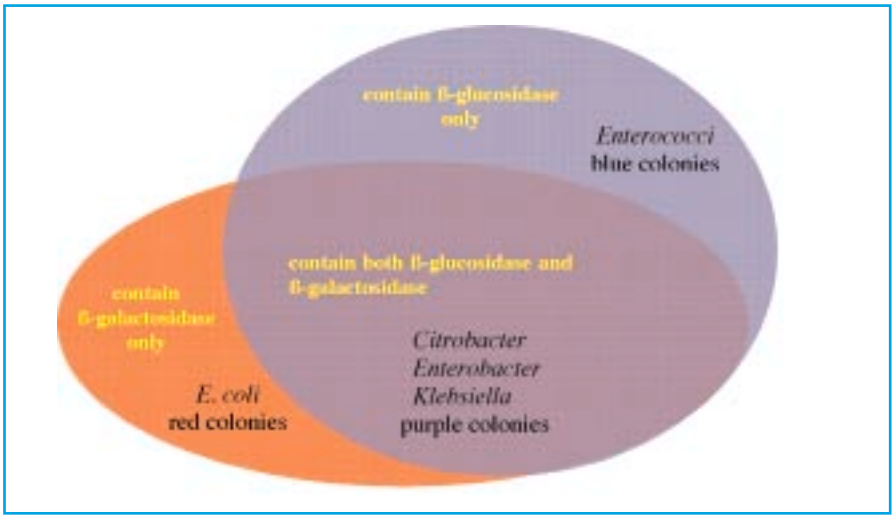

Figure 5. Species potentially isolated from urine streaked onto a non-selective medium containing 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside and 5-Bromo-6-chloro-3-indolyl-β-D-galactopyranoside.

Chromogenic agars

It is strange that given the widespread use of chromogenic substrates in biochemistry their application to microbiology did not really take off until the 1980s. This change was catalysed by the desire of water microbiologists for rapid screening procedures for the faecal indicator *Escherichia coli.* In 1988 the Association of Official Analytical Chemists (AOAC) gave first action status for the use of 4-Methylumbelliferone-β,D-glucuronide in Lauryl Sulfate Broth for the presumptive identification of *E. coli*[12]. This was based on the work by Feng and Hartman[13], who pioneered the use of this substrate for presumptive identification of *E. coli* in water and foods. Within a short time the AOAC then approved the Colilert system for use, a method for determining coliforms and *E. coli* in water. This system was based on the use of ONPG for the identification of β-Galactosidase-positive organism (coliforms) and the fluorogenic substrate 4-Methylumbelliferone-β-D-Glucuronide for the identification of *E. coli.* Approval by the AOAC for these two methods stimulated research into investigating the use of fluorogens and chromogens in culture media as a means of shortening the time for a presumptive identification of a variety of organisms.

Low specificities of conventional isolation media can result in prolonged testing of false positives with all the associated costs that this entails. The arrival of chromogenic substrates has both simplified and expedited screening for pathogens. The wide range of chromogenic hydrolase substrates that are now commercially available, particularly those based on indoxyl derivatives, has made possible the use of cocktails of substrates to simultaneously assay for a range of enzymes. The colour of a microbial colony is a mixture of the chromogens that have been released and reflects the hydrolase content of that organism. The Venn diagram in *Figure 5* shows an example of combining a β-galactosidase test for coliforms with a β-glucosidase test. It can be seen that combining two substrates increases the specificity of the medium, reducing the need for further testing.

In practice, various subtleties in these colours are often obtained in microbiological medium where media composition and pH can

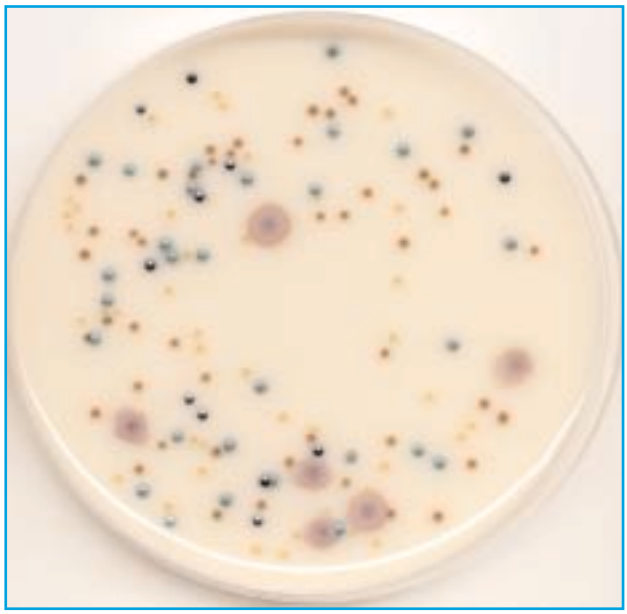

Figure 6. Strains of species of *Candida* plated onto medium containing different indoxyl substrates. *C. albicans*, green; *C. glabrata*, beige; *C. krusei*, purple/pink; *C. parapsilosis*, brown; *C. tropicalis*, dark blue.

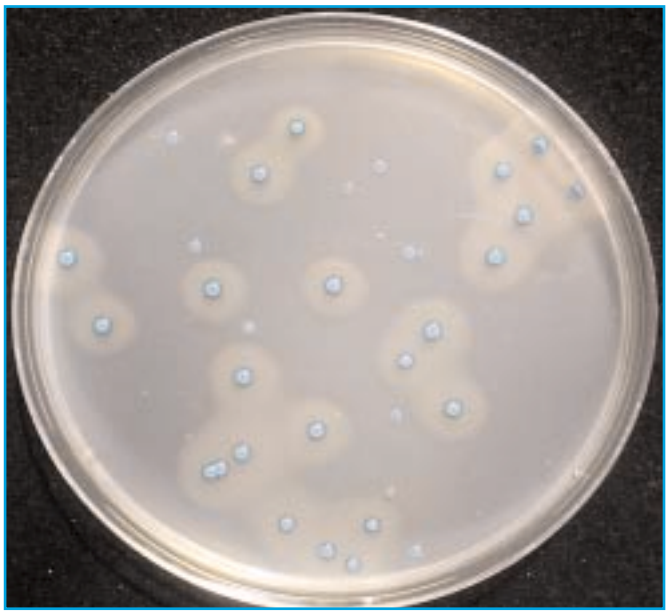

Figure 7. *Listeria* colonies appear as blue colonies due to the hydrolysis of 5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside. In addition, pathogenic *Listeria* utilise phosphatidyl choline to produce a halo around the colony.

affect the shade and spreading of the zones of colouration. This can add to the discriminatory ability of the medium and quite similar organisms can be differentiated (*Figure 6*).

Other biochemical tests may also be incorporated into chromogenic media to identify certain characteristics of the target organism. For example, when screening for pathogenic *Listeria* species the presence of phosphatidyl-specific phospholipase C may be detected by zones of clearing around colonies (*Figure 7*).

A major problem in both clinical and food microbiology is the doubtful specificity of tests for *Salmonella*. The organisms most likely to produce false positives on traditional isolation media such as Desoxycholate Citrate Agar, Mannitol Lysine Crystal Violet Brilliant Green Agar and Xylose Lysine Desoxycholate Agar are *Citrobacter* and *Proteus*. Unfortunately it is not possible to perform the two most useful enzyme tests (L-pyrrolidonyl arylamidase and 4-Nitrophenylalanine deaminase) for differentiating strains of these genera from *Salmonella* in an agar plate method. However, they can be used in the card method described earlier.

False-positive colonies can be rapidly and cheaply detected, allowing the user to concentrate their resources on any organisms that are negative for these tests.

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