How do we study microbes?

Hey, the cake I saved from last week is covered in spots of different shapes and colours. Are these microbes? How can we analyse them?

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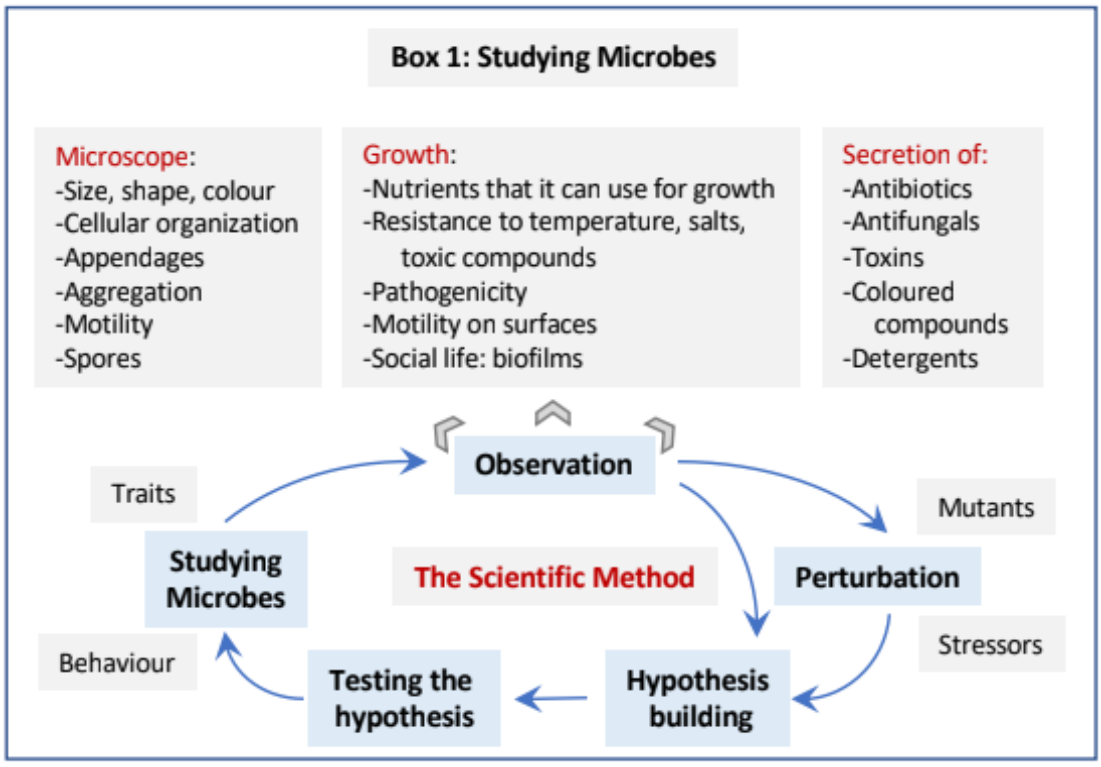
Storyline

Daily life provides plenty of evidence that microbes live all around us - even on us - but only occasionally do we really notice they are there, like when food goes off (even when kept in the refrigerator), when meat develops a fluorescent surface sheen, or when transparent bottles containing water develop a green film on the inner wall. But how do we know microbes are to blame? How can they grow under such conditions? Can we reproduce their growth in the laboratory? And how can we study their properties?

How do we study microbes?

1. ***The scientific approach to a big problem (microbes are not that easy to study!).*** If we suspect microbes are contaminating our food or growing in our drinking bottle, the first step in testing whether we are right is to make careful observations. Later we can try to cultivate the contaminating microorganisms. None of this, however, is straightforward. Microbes are usually very small (commonly just a few micrometres), which means we need a good microscope to see them. This is further complicated by the fact that it is hard to know exactly what we are looking at when we find them; many different microbes exist, and many of them look very similar. Culturing microbes can be tricky too since they rarely grow in isolation; rather, they tend to form communities composed of different species whose members interact and may even rely on each other in order to grow. Can we isolate *specific* types of microbes from these communities and study them on their own? Actually, we cannot really know in advance. We need to proceed step by step, first observing, then formulating hypotheses, and then designing experiments to test whether our hypotheses are correct. *That's the scientific method*, it's the way scientists approach problems.



2. ***Planning a properly designed experiment.*** When we want to analyse a microbe or a microbial community, we need to keep some things in mind:

   a. *The complexity of the sample*. Does it contain just one type of microbe (this can be the case with some clinical specimens, like blood, which ordinarily do not contain microbes except during an infection), or many different microbial species that together form a community (the usual case)?
   b. *Can these microbes be cultivated on laboratory media? Can we actually reproduce the environmental conditions under which they normally live? Can pure cultures of the individual species be obtained?* Sometimes none of this is possible, determining the kind of analyses we need to undertake.
   c. *How much detail do we need?* Do we just want to know what microbes are there and how they behave, or do we want to go further and understand how and why they behave the way they do?

As a general rule, if a problem is very complex, i.e., if it has many components and/or variables, we can approach it in a *reductionist* manner. To do this, we divide the problem into smaller parts and try solve each of these before gathering the individual solutions together to solve (we hope) the original, larger problem.

In our work we must always remember a *key* aspect of experimental science: *reproducibility*. A single measurement or observation is a good start, but that rarely provides certainty. Certainty derives from reproducibility: if you repeat an experiment several times and the result is always the same, you can be confident about that result. If the individual results are similar, but not identical, the degree of confidence in them that we can allow ourselves has to be determined by statistical calculations. The greater the similarity among replicas, the greater the confidence we can have. But what confidence level is acceptable? That depends on the problem faced and just how certain we really need to be.

We must also remember "controls", another *key* element of experimentation. Controls are samples of *known* composition or behaviour that are analysed in parallel with the *problem* samples. They tell us whether the technique being used is working correctly, or at least its degree of accuracy. Analytical techniques that provide accurate results are, of course, very important, but so too is the proper processing and interpretation of the data collected. Misinterpretations are always possible, leading us down the wrong path.

3. ***Starting our analysis. First observe the microbes, then perturb them and see how they react.*** OBSERVATION is the first step in analysing any microbe or microbial community. Watch the microbes, follow their behaviour, try to learn something about their habitat and their growth. Make measurements of variables that might be important (the temperature, humidity, the concentration of salts, etc.). This will give us an idea of the complexity of the problem. We can them make our first hypotheses, and design the first experiments that will test them and provide us with further information. The results of our first assays may validate our hypotheses, force us to modify them, or even reject them.

When the first rounds of observation/hypothesis/validation are complete, it is time to take the next step: PERTURBATION. Perturbation is used to obtain information on how something reacts to disturbance and may provide information on how it works. (E.g. if someone pokes you on the arm, do you poke back or move away? This perturbation gives information on your behavioural responses.) You could perturb the ecosystem, the habitat, or the microbe itself to test your hypothesis on how it all works. You could change the growth temperature, the composition of the growth medium, add compounds such as antibiotics, or, if possible, alter the genetic content of the microbe under study, inactivating particular genes, thus making

mutants (making mutants does not always work because some genes are dispensable or partially dispensable, but others are essential and cannot be inactivated). The results you obtain in this and further rounds of the same will either validate your hypotheses or tell you to reject or refine them, providing you a growing understanding of the microbe or microbial community that interests you.

Below are some examples that illustrate how to take on such an analysis. Some rely on observing the microbe or its environment. Others are more directed towards perturbation and seeing how things respond.

4. ***Key traits of microbes: how to study them.*** There are many types of microbe. They can vary greatly in size, complexity, lifestyle, nutrient requirements, and the ability to adapt to different environments, different temperatures, etc. The following lines discuss some of the key characteristics we will need to know.

a. *Complexity.* Microbes are usually unicellular, but can nonetheless be quite complex. All known forms of life can be grouped into one of the three great Domains of Life: the *Bacteria*, *Archaea* or *Eukarya* (viruses are a special case that do not fit into this scheme). In the members of *Eukarya* (known as eukaryotes), the genome is enclosed within a membrane, forming the nucleus. In *Bacteria* and *Archaea*, the genome is not enclosed by a membrane; there is no defined nucleus. These organisms are termed prokaryotes. All animals and plants are eukaryotic, but microbes can be either prokaryotic or eukaryotic. Eukaryotic microbes include fungi such as yeasts, unicellular algae and protozoa. Prokaryotes include all known bacteria, as well as the archaea; archaea are unicellular and share some properties of both bacteria and eukaryotes. A very good way to start when studying a microbe is to determine whether it is a eukaryote or a prokaryote. Eukaryotic cells are usually larger than prokaryotic cells, and can be easily seen under the light microscope. They also have characteristic "organelles" inside them that are absent in prokaryotic cells (although some bacteria have organelle-like structures; *exceptions always exist in biology!*). Some genes are useful markers of whether a microbe is a eukaryote, an archaea or a bacterium. The most useful of such gene codes for an RNA named 16S-RNA in bacteria and archaea, and 18S-RNA in eukaryotes. The sequence of this gene tells you to which group a microbe belongs (and can frequently even indicate the genus and species). Gene sequencing was not easy just a few decades ago, but today it is routine.

b. *Does the sample to study include just one type of microbe, or a community of different microbes?* Microbes rarely live in isolation; rather, communities of different microorganisms share a common living space (which complicates things). However, sometimes, under certain environmental conditions, a particular type of microbe can become very successful and eventually predominate. Light microscopy can provide a first clue in this regard: are all the cells very similar in size, shape and colour?

Nowadays it is possible to sequence *all* the DNA present in a given sample, without the need to cultivate the microbes in the laboratory. The sample might be a few grams of soil, or of sea water or fresh water filtered to concentrate the microbes. This discipline is called *metagenomics*. You start by purifying all the DNA in the sample, and then you sequence it. The result is a messy list of small DNA fragments of known nucleotide sequence. However, these fragments can be assembled into larger segments, then into genes, then into larger genome segments and, if possible, even into complete genomes. This requires the use of informatic methods (a discipline called *bioinformatics*). To investigate the number of microbes present in the sample we do not need to assemble their complete genomes. The same bioinformatic methods can sort out the number of different 16S-RNA and 18S-RNA genes present in the sample, therefore revealing its complexity. If we find just one type of 16S-RNA gene, we are

dealing with a pure culture of one type of bacterium or archaea. If several types are present, we have a community of different bacteria and/or archaea. If 18S-RNA genes are also detected, eukaryotes are also present (for example protozoa that feed on prokaryotes).

c. *Can a microbe be cultivated in isolation?* It is much easier to study a microbe if you can isolate it and culture it on (or in) laboratory culture media. A detailed description on cultivation procedures is explained in another Topic Framework. However, it is now believed that the majority of microbes cannot be cultivated in the laboratory. This is largely because they have growth requirements that either we do not know about or cannot provide in the laboratory. However, to really know whether we can cultivate a given microbe, we need to try. The first step is to take a tiny scrap of the sample under study (e.g., of the spoiled food or the fluorescent patina on the surface of the meat we talked about earlier), and inoculate it into what we think might be an appropriate growth medium. Now, there are *many* types of laboratory growth medium we could try. The so-called "rich media" include all sorts of nutrients (amino acids, sugars, vitamins, etc.), while other, more specific ones include just a single source of carbon and energy (for example a sugar such as glucose) and a buffered solution of salts. Every microbe has its own preferences.

Growth media can be either liquid, with the microbes thus cultured in flasks or bottles, or solidified with agar, providing a damp surface on which the microbes can grow. If the sample is diluted properly and spread onto the agar surface so that each single cell ends up lying far apart from next, and cultivation proceeds at the proper temperature (each microbe has its own temperature preferences), these cells may grow, divide and eventually form a colony large enough to be seen with the naked eye. If all the colonies on our agar plate look the same, the sample might be a pure culture. If the colonies that grow have different colours and morphologies, then the initial sample likely contained several different microbes. But remember, not all the microbes present in the sample may be represented; several may not have been able to grow on the agar surface because the conditions they require were not met. However, we can now take samples of the colonies we have grown, replicate them on new agar, and study the microbes that form them in isolation.

d. *What are the growth requirements of a given microbe?* Microbes require food to build up their cell mass and provide energy to keep their cellular machinery working (yes…life is a struggle against the laws of thermodynamics; it requires energy). Some microbes are very picky and can only use a narrow range of nutrients, while others are more versatile. For example, some bacteria are specialized in metabolising sugars, while others prefer amino acids (derived from proteins), fats, or even hydrocarbons similar to those present in crude oil. And certainly, there are plenty of bacteria that metabolize combinations of these compounds. In fact, microbes commonly use a wide range of nutrients as sources of carbon, nitrogen, sulphur, etc., and may obtain their energy not only from sugars and fats but in some cases even from inorganic compounds or sunlight. Those that use light are called photosynthetic microbes (photosynthesis first evolved in bacteria over 2000 million years ago; plants appeared much, much later, and acquired the trick from photosynthetic bacteria).

We also need to remember that environmental cues can put microbes under significant stress, and only those that can adapt will survive. Environmental factors such as the salt concentration, the temperature, the level of light radiation, or of oxygen, humidity, or the presence of toxic substances (including excessive concentrations of certain metals) can determine which microbes will survive and which will not. For example, some microbes are adapted to life in salty environments, and simply will not grow in freshwater, but some others have the opposite preference… and yes, some can manage in both. The same goes for temperature: some like it cold, some like it warm, and some like it really, really hot. Indeed,

microbes have been found in essentially all environments that contain liquid water, from some degrees below 0°C (salty water can still be liquid at temperatures slightly below 0°C) to over 100°C (under appropriate pressure, water can remain liquid at temperatures of about 110°C). The presence of very high concentrations of iron or arsenic, such as those found in the waters of the Rio Tinto River in Spain, also exerts a strong selective pressure, yet many microorganisms have adapted to life under these conditions.

Microbes have adapted to living in many different environments, even extreme environments. **(A)** A salty lake in the Atacama Desert (Chile); despite the very high salt concentration, microbes and crustaceans are present (and serve as food for flamingos). **(B)** The Rio Tinto river (Spain); these waters contain high concentrations of iron oxide (thus the red colour), as well other metals that are toxic to many forms of life. However, many microbes have adapted to existence in these waters, and are part of complex ecosystems. **(C, D)** Hydrothermal springs in the Azores Islands. These waters are very hot and rich in sulphur-containing compounds - but still home to certain types of microbe. Indeed, microbes have been isolated from many similar hot springs. (Photos by F. Rojo)

But can you know what growth requirements a given microbe has? First, you can make an educated guess based on the environment from which your original sample was taken, but only repeated experimentation will let you know for sure. You should try to cultivate your microbe under different conditions, with different nutrients, at different temperatures and salinities, with or without oxygen, etc. If you are lucky and patient enough, you might discover conditions under which your microbe can grow.

e. *What can I do if the microbe I am interested in cannot be cultivated in the laboratory, but metagenomics tells me that the microbe is present in a given sample?* In this case, you have to trust in bioinformatics. If your metagenomic results have provided us with the sequence of at least some of your microbe's genes, you can compare these sequences with those of other microbes for which genetic and physiological information is available in the scientific literature and genome data banks. Genes that share a significant sequence homology (i.e., their sequences are

similar) usually code for products with similar functions. But beware of misinterpretations! For example, two enzymes from two different types of microbe that show significant sequence homology will probably catalyse similar biochemical reactions - but perhaps in different metabolic pathways! Similarly, imagine that you find a gene in the microbe you are studying that codes for a protein very similar to another one you *know* to be present in a different, well-characterized microbe, and in which it is known to regulate the expression of another gene. You may deduce that the gene found in your metagenomic analysis also codes for a protein that regulates the same gene as in the well-characterized microbe. You will most likely be correct in the first assumption: both proteins will probably be regulators of another gene's expression. But you risk being wrong with the second assumption: they might not necessarily regulate the *same* genes in the two microbes.

f. *I have isolated a new microbe! Can it colonize plants, animals, or humans? And, if so, could its influence be beneficial or harmful?* This is an important question - and not so easy to answer. Some microbes can live associated in some way or another with plants or animals and create no problems at all; they may even be beneficial. For example, we all have an *entire microbial ecosystem on our skin* that protects us from incoming pathogenic microbes, and the digestion of food in all animals (including ourselves) is based on the action of a complex intestinal microbiota. And with respect to plants, many microbes can live on the surface of leaves, associated with the roots (e.g., in clover and chick peas they help the plant obtain the nitrates it needs), or even inside the vascular system, without causing any problems.

However other microbes have a more aggressive nature, and can 'feed' on animal or plant tissues, causing them harm. These fall into two groups: specialized pathogens, and opportunistic pathogens. Specialized pathogens can *only* survive by infecting a host, and are rarely found elsewhere. The immune system usually keeps them in check, but if it fails they may invade the tissues and organs, sometimes with fatal results. Opportunistic pathogens, in contrast, are not specialized and may be able to live in many different environments - with plants or animals (including humans) being possible habitats. These microbes are able to use many different compounds as nutrients, can withstand many harsh conditions, and are commonly found in soils and water, as well as in infected plants or animals. Again, the immune system usually keeps them in check and impedes their growth in tissues and organs, but if it fails, their ability to 'feed' on organic matter (i.e., our tissues) can make them very dangerous. Their low level of specialisation means they can often make use of a 'feeding' opportunity that results in them being pathogenic...hence the name opportunistic pathogen. Unfortunately, these microbes are commonly quite resistant to antibiotics.

If you have isolated a microbe and want to know whether it can be pathogenic, you should first make an educated guess, and then see if you are correct. For example, where did you isolate the microbe from? If it was from seawater, it will most likely be adapted to life under high salt concentrations, and our tissues would therefore not be an ideal habitat. But exceptions do exist. The only way to know is to see on what it will grow. Early rounds of investigation should include determining the temperature and salts necessary for optimal growth, and which compounds it can use as nutrients. If it can act as a human pathogen, it will probably grow at 37°C - our body temperature. Simple, standardized tests are now commercially available that will allow you to easily figure out which compounds a microbe can assimilate or modify, and which antimicrobial agent can inhibit its growth. The results give you a metabolic and an antimicrobial sensitivity pattern for the microbe, which can be compared with other *known* patterns for different microbial groups. This is fast and straightforward and is used for the rapid characterisation of microbes in hospitals. More complete methods are available too, although they are more work-intensive. The main one is, again, to isolate the microbe's DNA and

sequence the gene for 16S-RNA (prokaryotes) or 18S-RNA (eukaryotes), as explained above, and compare your results with those in public databanks (which nowadays harbour *huge* amounts of information); this will identify the known microbe most similar to yours. If there is a match, bingo! You have covered a huge distance towards knowing whether your microbe can be pathogenic. If there is no match, however, you might have a new microbe, and you will need to study it from the scratch. Just as described above, this will include analysing its behaviour (media in which it can grow, optimal temperature, salt concentration, its ability to infect plant leaves or animal tissues, etc.), sequencing its entire genome, and then comparing individual genes with those present in databases in order to identify your new microbe's closest relatives (and therefore what characteristics you might expect it to have). Certainly, it is a good idea to search for genes that are known to facilitate pathogenic behaviour, such as those coding for proteases (which slice up proteins), lipases (which cleave lipids), or one of the many toxins that pathogens produce.

5. ***Can a microbe modify the environment in which it lives?*** Ultimately, *all* living things influence and modify the environment in which they live. Microbes consume nutrients, and produce wastes. Depending on what a microbe consumes and excretes, its effect on the environment will differ. For example, the consumption of sugars will generate CO_2 as waste, which will likely acidify the growth medium. But if cells are using amino acids as food they will also excrete ammonia, making the surrounding medium more alkaline. Many prokaryotic microbes produce methane (or other gases with a powerful greenhouse effect) rather than CO_2; the microbes in the gut of ruminants are a good example, but there are many other environments that harbour methane-producing microbes.

The products consumed and secreted by a microbe can be detected and their rates of consumption or secretion measured, provided we operate in a closed system such as a flask or a fermenter in which we can carefully measure what is consumed and what is produced (even gases). For a few model microbes for which a great deal of information is available, bioinformaticians have constructed what we call "metabolic models". These are basically a list of biochemical reactions known to occur in the microbe. If a model is provided with enough good-quality information regarding which compounds the microbe consumes, at what rate, and the rate of increase in the culture biomass, it can predict the metabolic reactions most likely driving its metabolite fluxes, and tell us which compounds it will secrete. The more complete the model is (i.e., the more biochemical reactions you tell it that your microbe does), and the better the experimental data provided, the more accurate its predictions will be. But, of course, models only make predictions: they just let us know which compounds we should try to measure experimentally and quantify in the growth medium. Metabolic models are very useful, and can provide good predictions if the input data are accurate and complete, but we always need to test their predictions.

6. ***Can the genome of a microbe be modified? Can we make mutants***? A very powerful way to study microbes, their properties, their ability to interact with the environment, and the function of given genes, is to generate mutants of the genes of interest, thus modifying their function or inactivating them. For example, if we eliminate a gene in a bacterium resistant to a given antibiotic, and the consequence is that it becomes sensitive to that antibiotic, then we have found a gene likely involved in its original resistance to that antibiotic (knowing *how* that gene confers resistance against the antibiotic is something that will need further work). When making mutants, however, the aim is not always to eliminate a gene: sometimes it is better to alter it, and thus modify the protein it encodes. We could also alter the expression of that gene

and thus modify the amount of protein produced. If any of these modifications produces a change in the behaviour of the microbe, it gives us information on the function of the gene. The modification of specific genes in well-known microbes can also help us direct them to perform tasks that are useful to us; this is the basis of *biotechnology*. For example, a gene might be modified to allow a microbe to more easily degrade a toxic compound (the basis of *bioremediation*), or to produce and secrete a valuable product that can be purified and marketed.

There are two approaches we can take to modifying or inactivating genes. One is not gene-specific, and is based on treating the microbe with an agent that modifies the genome at random, for example a mutagenic compound (a chemical) or radiation of sufficient energy (e.g., UV light). The other relies on techniques that allow the mutation of just a specific gene of interest.

Random mutagenesis may simultaneously cause mutations in several genes (with stronger treatments generating more mutations per genome). However, too many mutations can compromise a cell's viability. Moreover, finding out whether you have managed to mutate a gene of interest to you is not straightforward; you have to screen for it based on the loss (or perhaps gain) of a characteristic that you can observe easily. You could, say, select for genes that when inactivated increase or reduce the resistance of a bacterium to a given antibiotic. However, the larger the genome of the analysed microbe, the lower the chance that a mutation will occur in a gene of interest rather than in a non-related gene.

Targeting mutations to a specific gene is far more useful, but has its own problems. Many techniques are available for modifying or inactivating a specific gene, but some are very sophisticated. And not all microbes are equally amenable to genetic manipulation. A mutant gene is usually constructed *in vitro*, outside the original microbe of interest, using a plasmid (an independent self-replicating circular piece of DNA) to hold and transport it, and a model organism to host it (typically a laboratory strain of *Escherichia coli*). The modified gene can then be introduced into the genome of the microbe under study. The ease of this step depends on whether the microbe is amenable to genetic manipulation or not. Indeed, microbes have defence mechanisms to minimize the entry of foreign DNA (for example a virus or an undesired plasmid that may behave parasitically), and such barriers are more effective in some microbes than others. Many of these barriers have been disabled in the model bacterial strains routinely used in research laboratories. If foreign DNA containing a modified gene can be introduced into the microbe we are studying, then we might be able to exchange the original gene for the modified (mutant) one. It helps to impose a strong selection pressure that will select for the presence of the modified gene. It is not the aim of this chapter to make a list of all the techniques available for manipulating genes, but a glimpse of what can be done, and what results might be expected, is provided below.

a. *Gene inactivation. A gene* can be inactivated by insertion of a DNA segment within it, thereby interrupting the coding sequence, a bit like changing the sentence: *I would like an egg for breakfast* to *I would like an egg* XYZ *for breakfast.* This introduced segment will usually contain a marker gene, for example that confers resistance to an antibiotic to which the microbe is sensitive. This allows for the rapid selection of the mutant. Inactivation can also be achieved by eliminating a small internal segment of the gene, or even the complete gene, using a variety of sophisticated techniques.

b. *Gene modification*. Sometimes we want to modify the sequence of a gene to make a specific mutant protein. This is done to analyse the function of a given region of the protein it codes for, or of a given amino acid residue in that protein. For this, we can construct a mutant gene using either genetic engineering techniques or by chemically synthesizing a custom-made

segment of DNA. Modifications may include the introduction of a stop codon upstream of the natural one (in this case the protein produced will lack a terminal segment and will be shorter, e.g *I would like an egg for*), or the alteration of one or more codons (in this case the protein produced will have site-directed mutations at amino acid residues of our choice, e.g. *I would like an ugg for breakfast).*

c. *Modification of gene expression.* Here we do not modify the gene itself but rather its expression (increasing or reducing it) in the hope of seeing an effect that reveals the function of that gene. Again, there are several techniques for doing this.

Potential Implications of our decisions regarding microbes

1. *Individual*

a. *Should we be afraid of microbes*? Certainly, microbes can grow on our food, but in some cases this may be beneficial (yoghurt, kefir, bread, cheese) and in others potentially harmful (when pathogenic or toxin-producing bacteria are present). Most bacteria are harm*less*, but avoiding those that can be harm*ful* can preserve our health.

b. *Should I clean this surface?* Many microbes colonize surfaces, forming biofilms, but to what extent do we need to clean them? For example, it would be very important to clean them from dairy industry equipment, but not so important to sterilize your hiking boots.

c. *Should I keep my food in the refrigerator?* Many microbes, and certainly most of those that can be pathogenic, do not grow well below 10°C, although exceptions do exist (there are a few pathogenic bacteria that can grow at 5°C). Cooking or sterilizing food is a good solution. But, when it comes to fresh food, we need to ask whether it is advantageous to add compounds that prevent the growth of microbes? We have to balance the benefits and pitfalls.

2. *Community-National policies*

a. Regulating food and drink safety.
b. Health costs of living in environments contaminated with pathogenic microbes.
c. Valuing ecosystem services provided by microbes.

Pupil Participation

1. Class discussions

a. Discuss the process of the scientific method for solving questions

b. Discuss whether modifying the genome of a microbe can be dangerous, neutral or beneficial to society

c. When making mutants of a given microbe, is it better to use random mutagenesis or site-directed mutagenesis? Can we always choose?

d. Microbes good or bad?

e. Where do you think microbes can live?

f. Why are microbes important in biotechnology? Can you give an example?

g. Have you ever seen spoiled food, green water or any other indicator of the presence of microbes in everyday life?

2. Exercises

a. List the steps you need to take to show that a microbe is responsible for the spoilage of mayonnaise.

b. What steps should you take to study a microbe implicated in a disease?

c. How many pollutants do you know? If you wanted to bioremediate one of them, using a microbe that can assimilate the pollutant, where could you find that kind of microbe? (Make an educated guess…)

d. Starting from a bacterium sensitive to an antibiotic, think of the simplest way to isolate a mutant that has acquired resistance to that antibiotic.