

cells, the environment can alter when bacteria invade due to changes in host gene expression. Since environmental conditions affect bacterial growth, metabolism and regulation of gene expression (Busby *et al.* 1998; Marshall *et al.*, this issue) we should expect bacteria taken from infected animals to be different, in some respects, from those grown *in vitro*, a fact now well established for many pathogenic species (Smith 1990, 1996).

Clearly, the activities of bacteria *in vivo* must be explored for a fuller appreciation of pathogenicity. There are three aspects to the full picture. First, there are observations on the bacteria themselves and identification of virulence determinants formed by them at different stages of infection. Second, there is recognition of host factors that affect bacterial behaviour and production of virulence determinants. Third, there is investigation of the underlying metabolic interactions between bacterial and host factors. The first aspect is relatively easy to accomplish and recently new methods for doing so have been devised. The second is not easy to achieve because of the complexity of the environment *in vivo* and the fact that it changes as infection proceeds. Nevertheless, there is relevant information in the literature and some new methods have been evolved. The final aspect is very difficult to study effectively *in vivo*; some progress might be made if events *in vivo* can be simulated *in vitro*. It is not surprising that the first aspect receives most attention. Indeed, one can understand an attitude to concentrate solely on bacterial properties because the environment *in vivo* and its influence are too complex to analyse properly. However, this leaves part of the story untold.

The new methods for studying bacterial pathogens *in vivo* are listed in Table 1. Some require the pathogen to have robust genetics that are easily manipulated, which is not always the case, e.g. for *Campylobacter jejuni*. The objectives of this Discussion Meeting were to describe these methods and to show how they, and a recent surge in conventional studies, are advancing knowledge. This paper sets the scene by posing some questions and suggesting, with examples, methods whereby answers may be forthcoming.

2. QUESTIONS ABOUT THE DETERMINANTS OF BACTERIAL GROWTH *IN VIVO*

The multifactorial nature of pathogenicity means that the determinants of all five requirements are essential for its manifestation. However, growth

Table 1. New methods for studying bacterial behaviour *in vivo*.

Function	Method	Principle	Reference
Following infection in animals	Confocal laser scanning microscopy (CLSM)	Possible to examine a few bacteria in thick slides	Richter-Dahlfors <i>et al.</i> 1997
	Fluorescence-activated cell sorting (FACS)	Rapid identification of host cells containing fluorescent bacteria	Valdivia & Falkow 1997a
	Laser microprobe mass spectrometry	Measuring viability of bacteria in biopsies by Na ⁺ /K ⁺ ratios	Haas <i>et al.</i> 1993; Seydel <i>et al.</i> 1992
	Photonic and radio detection of pathogens <i>in vivo</i>	Pathogens made bioluminescent by a luciferase or radiolabelled by technetium-99 m	Contag <i>et al.</i> 1995; Perin <i>et al.</i> 1997
Measuring environmental parameters <i>in vivo</i>	Quantitative fluorescence microscopy use of reporter genes	Measurement of fluorescing dyes that react to environmental factors <i>LacZ</i> fusions to genes that respond to certain levels of compounds in the environment	Akins <i>et al.</i> 1995; Aranda et al 1992 Garcia-del Portillo <i>et al.</i> 1992; Pollack 1986
	X-ray microanalytical electron microscopy		Morgan 1985; Spencer <i>et al.</i> 1990
Detection of genes expressed <i>in vivo</i>	<i>in vivo</i> expression technology (IVET)	Genes expressed <i>in vivo</i> provide promoters for various reporting systems	Camilli <i>et al.</i> 1994; Camilli & Mekalanos 1995; Heithoff <i>et al.</i> 1997; Lowe <i>et al.</i> 1998; Mahan <i>et al.</i> 1994, 1995; Wang <i>et al.</i> 1996a, b; Young & Miller 1997
	Differential fluorescence induction (DFI)	Promoters of genes expressed <i>in vivo</i> drive expressions of green fluorescent protein	Valdivia & Falkow 1996, 1997a

Table 1. (Continued)

Function	Method	Principle	Reference
	Differential display of cDNAs	cDNAs prepared from mRNAs of genes expressed <i>in vivo</i> and compared with cDNAs from organisms <i>in vitro</i>	Abu-Kwaik & Pedersen 1996; Plum & Clark-Curtiss 1994
	Reaction with antibodies produced by infection	Reaction of products of gene libraries with antibodies evoked by infection compared to those of antibodies formed against killed bacteria	Akins <i>et al.</i> 1995; Suk <i>et al.</i> 1995; Wallich <i>et al.</i> 1995
	Labelling proteins with diaminopimelate	Diaminopimelate used by bacteria and not by host cells	Burns-Keliher <i>et al.</i> 1997
Direct identification of virulence genes	Signature-tagged mutagenesis (STM)	Non-recovery from animals after inoculating individually tagged insertion mutants indicates genes required for infection	Hensel <i>et al.</i> 1995; Chiang & Mekalanos 1998; Coulter <i>et al.</i> 1998; Mei <i>et al.</i> 1997; Shea <i>et al.</i> 1996
	Virulence complementation	Complementation of avirulent strains by gene libraries from virulent strains	Collins 1996; Pascopella <i>et al.</i> 1994
Global analysis of potential gene expression	Complete genome sequencing	Aids the identification of new genes expressed <i>in vivo</i>	Strauss & Falkow 1997; Tang & Holden 1999
	Chip technologies	Microarrays of probes allow monitoring of expression of many genes in parallel	De Saizieu <i>et al.</i> 1998; Lockhardt <i>et al.</i> 1996; Ramsay 1998; Schena <i>et al.</i> 1996

holds the primary position because without it other determinants would not be formed. This is the first reason for dealing with growth separately. The second is that the new methods for recognizing genes expressed *in vivo*

(Table 1) are underlining its importance. Many of the genes detected are involved in the acquisition of nutrients and their metabolism, e.g. members of the 100 or more genes demonstrated by *in vivo* expression technology (IVET) for infections of *Salmonella typhimurium* in mice and macrophages (Heithoff *et al.* 1997). Finally, compared with other aspects of virulence, growth and metabolism have been neglected because it is difficult to do meaningful experiments. A discussion of these difficulties and possible ways of solving them could encourage more work in the area.

Perhaps the first point to emphasize is that it is not just growth, but rate of growth, that is important in pathogenicity. On epithelial surfaces, receptors for some bacterial adhesins are present in mucus and could delay contact between the pathogen and the surface. Their influence can be overwhelmed by rapid and substantial bacterial growth in mucus (McCormick *et al.* 1988; Mantle & Rombough 1993). At primary lodgement, the few bacterial invaders must multiply rapidly to replace losses inflicted by the powerful host defences of the inflammatory response. In acute disease, rapid growth of the pathogen in tissues is needed to produce harmful effects before a protective immune response is mounted. In chronic disease, slow growth at all stages may lead to less stimulation of immune responses. To form carrier states, a resistant stationary phase of the pathogen (Kolter *et al.* 1993; Kolter 1999) may be an advantage. These different growth rates will be determined by prevailing environmental conditions, which will also influence the size of pathogen populations that can be sustained by different tissues.

The important questions regarding bacterial growth *in vivo* are as follows. What is the pattern of a developing infection and growth rates and population sizes at different stages? What nutrients become available or depleted as infection proceeds and how do they relate to growth rates and population size? How are the nutrients metabolized and by what bacterial determinants? How do bacteria handle nutrient deficiencies and antagonistic biochemical conditions? They are discussed in two sections, bacterial activities and host factors.

(a) *Bacterial activities*

The classical method of following pathogenesis is to take samples of body fluids and tissues during the course of infection, either from live animals

or those killed at intervals, and then to examine them outside the host by *in vitro* methods. The latter include total and viable counts, and light and electron microscopy. Recently, confocal laser scanning microscopy (CLSM), fluorescence-activated cell sorting (FACS), and laser microprobe mass spectrometry (Table 1) have added new dimensions to these classical methods. For example, CLSM of immunostained sections of livers of mice infected with realistically small doses of *S. typhimurium* showed that the pathogen resides intracellularly in macrophages and is cytotoxic to them (Richter-Dahlfors *et al.* 1997), as occurs with cultured macrophages (Chen *et al.* 1996). Hence, efforts to identify the molecular determinants of cell culture cytotoxicity are now relevant to behaviour *in vivo*. The major advance has been to use non-invasive methods such as photonic imaging and radiolabelling (Table 1) for following the pattern of infection. For example, photonic imaging provided a surprise about salmonellosis of mice; after oral infection, the organisms were concentrated in the caecum rather than the ileum (Contag *et al.* 1995).

Increases or decreases in bacterial populations have been measured by counting bacteria in blood, lymph glands, spleen, liver and other relevant tissues, e.g. Peyer's patches (Curtiss *et al.* 1988; O'Callaghan *et al.* 1988). Now, the new non-invasive methods can be used for evaluating population changes in different tissues.

In considering growth rates *in vivo*, it should be remembered that population size is the result of bacterial growth and destruction or removal by the host. If a population increases rapidly, there is no doubt that bacteria are multiplying rapidly. But, the precise growth rate is unknown because, although dominated by the growing pathogen, host defence will have some effect. When the population increases slowly, or even decreases, as happens early and late in the disease process, multiplication rates are not clear. A rapid growth rate may be masked by an equally quick destruction by the host. Certainly, a stationary population does not necessarily mean that the pathogen has stopped growing.

Methods for measuring doubling times in tissues are available. The first methods relied on a genetic marker distributing to only one of two daughter cells in each succeeding generation. The proportion of the bacterial population carrying non-replicating markers, examined at intervals during infection, revealed the number of preceding generations (Maw & Meynell 1968; Hormaeche 1980). The method was used for infections of *E. coli* and

S. typhimurium in mice but its scope was limited by the need for a non-replicating marker. The next method, used for mice infected with *E. coli* and *Pseudomonas aeruginosa* (Hooke *et al.* 1985; Sordelli *et al.* 1988), could have wide application. Growth rates were calculated from increases in ratios of wild-type organisms (which multiply *in vivo*) to temperature-sensitive mutants (which should not multiply *in vivo*) during the course of infection. Unfortunately, this method has not been exploited. Recently, a combination of the two methods has been used to compare growth rates in mice of virulent and attenuated strains of *S. typhimurium* (Gulig & Doyle 1993). The marker, inherited by only one of the progeny on division *in vivo*, was the temperature-sensitive Cm^r plasmid pHSG 422, which is maintained on replication at 30°C but not at 37°C. Overall, these methods indicated that growth *in vivo* was slower than *in vitro* in some cases (Maw & Meynell 1968; Hormaeche 1980) and similar in others (Hooke *et al.* 1985; Sordelli *et al.* 1985). But, their use has been limited. Since the 1960s, when the subject was first raised, only three pathogens, *E. coli*, *S. typhimurium* and *P. aeruginosa*, have been examined.

In view of the importance of growth rates in pathogenicity, it would be a great advance if an easily used, non-invasive method for measuring them *in vivo* could be devised. If a method became available, particular attention should be given to growth rates in the early, crucial stage of infection, which are obscured by the bactericidal effects of host defences. Also, the possible occurrence of non-growing bacterial populations in persistent infection and carrier states should be investigated, in view of the knowledge accumulating about stationary bacterial populations *in vitro* (Kolter *et al.* 1993; Kolter 1999).

(b) *Host factors*

The first method to recognize nutrients that might determine growth *in vivo* relies on the fact that most key nutrients, e.g. iron, will also be necessary for growth *in vitro*. Hence, the first step is to grow the pathogen in a defined medium and observe the effects of deleting specific nutrients. Then, the presence *in vivo* of the identified nutrients can be ascertained. Much information on sugars, aliphatic-, hydroxy- and long-chain fatty acids, amino acids, purines, pyrimidines, vitamins and metal ions in blood, body fluids, neutrophils, macrophages and other tissues is known from physiological,

pathological and pharmacological studies (Lentner 1981, 1984). Also, tissue samples can be analysed by established biochemical methods.

Some key nutrients may not be revealed by these studies *in vitro*. Erythritol is used preferentially by *Brucella abortus* in a medium containing glucose (Anderson & Smith 1965). It is concentrated in the placenta, foetal fluids and chorions of pregnant cattle, and during brucellosis promotes infection of these tissues leading to abortion (Keppie *et al.* 1965; Smith *et al.* 1962; Williams *et al.* 1964). Its importance in the metabolism of *B. abortus* was discovered by noting growth stimulation when foetal fluids or placental extracts were added to cultures *in vitro* and then purifying the stimulant. This procedure could be applied to other pathogens, particularly those that show tissue tropism in disease.

Auxotrophic mutants of pathogens can be used to check the availability of specific nutrients *in vivo*. For example, auxotrophs unable to synthesize p-aminobenzoic acid, purines, thymine and histidine have been prepared from *Salmonella typhi*, *S. typhimurium* and *Shigella flexneri* (Ahmed *et al.* 1990; Curtiss *et al.* 1988; Fields *et al.* 1986; Karnell *et al.* 1993; Leuing & Finlay 1991; Levine *et al.* 1987; O'Callaghan *et al.* 1988). They have low virulence for mice, rabbits, monkeys or man due to impaired growth *in vivo*, indicating that p-aminobenzoic acid, purines, thymine or histidine are absent or scarce in these animals. Similar auxotrophs for other nutrients could be prepared from different pathogens. The absence or presence of the specific nutrients would be indicated by comparing their multiplication rates *in vivo* with those of wild-types. This method reveals deficiencies in nutrients that may be required by other pathogens. It does not provide information on nutrients that the wild-type uses *in vivo* to synthesize the particular metabolite required by the auxotroph.

Another method for recognizing key nutrients of growth *in vivo* covers the possibility that they may not be the same as those needed for growth *in vitro*. Bacteria grown *in vivo* can be investigated by the new methods (Table 1) for genes that code for enzymes involved in acquiring and metabolizing nutrients. The nature of these enzymes will indicate nutrients used *in vivo*. In a signature-tagged mutagenesis (STM) study of staphylococci in infected mice, a prominent identified virulence gene (i.e. one which when mutated results in reduced virulence) coded for a proline permease, indicating that scavenging for proline is essential for virulence (Schwan *et al.* 1998).

After key nutrients have been identified their concentrations in the tissues can be obtained from the literature (Lentner 1981, 1984) or measured by appropriate methods. The latter is relatively easy for normal uninfected tissues but monitoring nutrient concentration in infected tissues and their changes as disease progresses is extremely difficult, even if good animal models are available.

Measuring environmental parameters within infected cells is not easy but some progress has been made using new methods. Quantitative fluorescence microscopy (Table 1) has been used to measure intraphagosomal pH in macrophages (Aranda *et al.* 1992) and LacZ reporter genes (Table 1) have been used to indicate Ca^{2+} , Fe^{2+} and Mg^{2+} levels in tissue culture cells (Pollack *et al.* 1986; Garcia-del Portillo *et al.* 1992).

The relevance of identified nutrients to infection *in vivo* can be tested by two methods. The virulence of the pathogen may be enhanced by injecting additional nutrient. Also, reduced virulence of mutants unable to use it could be investigated. If it is irreplaceable, e.g. iron, these mutations will usually be lethal. If, however, the nutrient is preferred but replaceable by another, e.g. erythritol by glucose for *B. abortus* (Anderson & Smith 1965), then the required mutant might be obtained.

Some biochemical conditions existing *in vivo*, which might have adverse effects on growth, e.g. high osmolarity, low pH and anaerobic conditions, can be identified and quantified by reference to the literature or by analysis. Antagonistic influences may also be indicated by the functions of genes whose expression *in vivo* is detected by the new methods (Table 1), e.g. those that deal with variations in osmolarity.

The final questions posed at the beginning of this section — How are the nutrients metabolized and by what bacterial determinants? How do bacteria handle nutrient deficiencies and antagonistic biochemical conditions? — are extremely difficult to answer. Trying to investigate the metabolism of pathogens growing in infected tissues is well nigh impossible. If some aspect of growth *in vivo*, such as doubling time or population size, could be simulated *in vitro* by culturing the pathogen in a medium to which nutrients known to be important for growth *in vivo* are added at the appropriate concentrations, some meaningful observations could be made by established methods of bacterial physiology. Similarly, the effects of adverse conditions could be investigated provided the phenomenon *in vivo* could be simulated

in vitro. In both cases, the enzymic products of genes shown to be expressed *in vivo* by the new methods (Table 1) should be kept in mind. Also, the complete genomes of pathogens will reveal their overall metabolic potential, e.g. the presence, absence or incompleteness of a citric acid cycle (Huynen *et al.* 1999).

In view of the complexity of the experimental systems, it is hardly surprising that there has been little progress in answering questions on the nutrients and metabolism that underpin bacterial growth *in vivo*. However, there are a few examples showing that answers can be obtained. The best is the acquisition of iron by pathogens *in vivo*. Iron is an essential nutrient for all bacteria. First, it was shown that availability of iron *in vivo* is restricted by chelation to host transferrin and lactoferrin, and that injection of iron salts enhanced virulence of many pathogens in various animal models (Bullen 1981). Then, molecular studies were conducted *in vitro* under iron-limiting conditions. These showed that different pathogens adopt numerous strategies to overcome iron restriction (Weinberg 1995). In some cases, siderophores are excreted, which chelate iron and return to bacteria via specially induced cell-surface protein receptors (Brown & Williams 1985; Weinberg 1995). After internalization, the siderophores give up their iron under the influence of reductases (Halle & Meyer 1992). In other cases, transferrin-bearing iron interacts with cell-wall protein receptors and iron is delivered into the bacteria (Cornelissen *et al.* 1992; Anderson *et al.* 1994). In both cases, the cell-wall receptors were shown to be present on bacteria in patients or infected animals (Brown & Williams 1985; Cornelissen *et al.* 1992; Smith 1990, 1996). Finally, mutants deficient in the determinants of iron acquisition were shown to be attenuated in virulence tests, e.g. a gonococcal mutant deficient in the transferrin receptor was less infective for human volunteers than the wild-type (Cornelissen *et al.* 1997).

Two other examples relate to tissue localization by pathogens. Urea is a growth stimulant for *Proteus mirabilis*, which causes severe kidney infections (Braude & Siemienski 1960). The fact that this growth stimulation contributes to localization in the kidney was supported by the impaired growth in the kidneys of mice of a urease-deficient mutant (MacLaren 1968). Also, the following evidence supports the role of erythritol in stimulating massive growth by *B. abortus* in foetal tissues of cattle, resulting in abortion (Keppie *et al.* 1965; Smith *et al.* 1965; Williams *et al.* 1964). Injections

of erythritol enhanced infection of *B. abortus* in newborn calves. Erythritol analogues inhibited growth of *B. abortus* *in vitro* and *in vivo*. A strain (S19) of *B. abortus* unable to use erythritol did not cause abortion.

Finally, there are results emerging from use of the new methods of detecting gene expression *in vivo*. Two previously unrecognized genes of *E. coli*, *guaA* and *argC*, induced in urine appear important in uropathogenesis (Russo *et al.* 1996). Urine contains no guanine and only low levels of arginine and the induced genes allow *E. coli* to synthesize them. Deletion mutants do not grow in urine and in mice are less virulent than the wild-type. In addition to these genes, the osmoregulatory transporter ProP, coupled with osmoprotective betaine, allow *E. coli* to grow in human urine and to colonize the urinary tract of mice (Culham *et al.* 1998). Turning to *V. cholerae*, STM identified an attenuated biotin auxotroph from the intestine of infected mice (Chiang & Mekalanos *et al.* 1998). This suggested that biotin synthesis is a virulence attribute and that there was little available biotin in the infant mouse intestine, a fact supported by enhanced colonization when biotin was added to the inoculum.

3. QUESTIONS ABOUT PRODUCTION *IN VIVO* OF DETERMINANTS OF MUCOSAL COLONIZATION, PENETRATION, INTERFERENCE WITH HOST DEFENCE AND DAMAGE TO THE HOST

Far more is known about these determinants than those responsible for growth.

(a) *Bacterial activities*

The fact that environmental conditions *in vivo* differ from those *in vitro* and change as infection proceeds has the following implications. First, some putative virulence determinants indicated by experiments *in vitro* may not be formed *in vivo*, and even if they are, they may not be necessary for virulence. Second, some determinants formed *in vivo* may not be produced *in vitro*. Third, the complement of determinants may change as infection proceeds and different anatomical sites are affected. The questions relate to the validity of these implications.