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Questions about the behaviour of bacterial pathogens *in vivo*

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Bacterial pathogens cause disease in man and animals. They have unique biological properties, which enable them to colonize mucous surfaces, penetrate them, grow in the environment of the host, inhibit or avoid host defences and damage the host. The bacterial products responsible for these five biological requirements are the determinants of pathogenicity (virulence determinants). Current knowledge comes from studies *in vitro*, but now interest is increasing in how bacteria behave and produce virulence determinants within the infected host. There are three aspects to elucidate: bacterial activities, the host factors that affect them and the metabolic interactions between the two. The first is relatively easy to accomplish and, recently, new methods for doing this have been devised. The second is not easy because of the complexity of the environment *in vivo* and its ever-changing face. Nevertheless, some information can be gained from the literature and by new methodology. The third aspect is very difficult to study effectively unless some events *in vivo* can be simulated *in vitro*.

The objectives of the Discussion Meeting were to describe the new methods and to show how they, and conventional studies, are revealing the activities of bacterial pathogens *in vivo*. This paper sets the scene by raising some questions and suggesting, with examples, how they might be answered.

Bacterial growth *in vivo* is the primary requirement for pathogenicity. Without growth, determinants of the other four requirements are not formed. Results from the new methods are underlining this point. The important questions are as follows. What is the pattern of a developing infection and the growth rates and population sizes of the bacteria at different stages? What nutrients are present *in vivo* and how do they change as infection progresses and relate to growth rates and population sizes? How are these nutrients metabolized and by what bacterial mechanisms? Which bacterial processes handle nutrient deficiencies and antagonistic conditions that may arise? Conventional and new methods can answer the first question and part of the second; examples are described. The difficulties of trying to answer the last two are discussed.

Turning to production *in vivo* of determinants of mucosal colonization, penetration, interference with host defence and damage to the host, here are the crucial questions. Are putative determinants, which have been recognized by studies *in vitro*, produced *in vivo* and are they relevant to virulence? Can hitherto unknown virulence determinants be recognized by examining bacteria grown *in vivo*? Does the complement of virulence determinants change as infection proceeds? Are regulatory processes recognized *in vitro*, such as ToxR/ToxS, PhoP/PhoQ, quorum sensing and type III secretion, operative *in vivo*? What environmental factors affect virulence determinant production *in vivo* and by what metabolic processes? Examples indicate that the answers to the first four questions are 'yes' in most but not all cases. Attempts to answer the last, and most difficult, question are also described.

Finally, sialylation of the lipopolysaccharide of gonococci *in vivo* by host-derived cytidine 5′-monophospho-N-acetyl neuraminic acid, and the effect of host lactate are described. This investigation revealed a new bacterial component important in pathogenicity, the host factors responsible for its production and the metabolism involved.

Keywords: bacteria; pathogens; *in vivo*; gonococci; sialylation; lipopolysaccharide

1. INTRODUCTION

Pathogenicity (virulence) is the capacity to cause disease. Bacterial pathogens form only a small part of the bacterial world but they attract the most attention. They have unique biological properties, which enable them to enter the tissues of man or animals and cause sickness and sometimes death. These biological properties are indicated by the progression of the disease process. The skin is a formidable barrier against bacterial attack. It can be breached by gross trauma or vector bite and a few pathogens, e.g. staphylococci are able to exploit small abrasions to cause skin infections. However, the usual route of entry for most pathogens is not through the skin but over the internal surfaces of the respiratory, alimentary or urogenital tracts. Initially, only a small number of bacteria are deposited on these mucous surfaces, so the first requirement for pathogenicity is survival and growth on them. Survival entails competition with commensals that normally inhabit these surfaces, penetration of mucus

that covers them and adherence to epithelial cells. Next, most pathogens need to penetrate into the tissues to be effective, although some, like *Vibrio cholerae*, can cause disease while remaining on the mucous surface. Penetration may be achieved by entry into and egestion from epithelial cells, by passage between them or destruction of them. The third requirement is the ability to grow and multiply in the environment of host tissues, otherwise the pathogen cannot cause harm. On the mucous surfaces and within the tissues, pathogens have to contend with antibacterial substances in body fluids and within the cells they infect. Also, there are polymorphonuclear (PMN) phagocytes and macrophages that can ingest and kill them. These defence mechanisms, which act against any invading pathogen, are present at the site of infection and, within a few hours, are reinforced by the inflammatory response. Clearly, ability to withstand these host defences is the fourth requirement for pathogenicity. This quality is needed again as bacteria spread through the lymphatic system into the bloodstream, where many macrophages line the vessels of the lymph nodes, spleen and liver. A few days after initial infection, the pathogen faces an even greater obstacle, the specific immune response. To survive, it must either suppress or circumvent antibody- and cell-mediated immunity. Finally, entry and growth in the host and inhibition of host defence are not enough. To be pathogenic, the bacteria must damage the host, thus causing disease. This can be achieved directly either by production of toxins or lysis of host cells by intracellular bacteria. It can also be accomplished indirectly by stimulation of host cytokines or immunopathology.

To summarize, for the many pathogens that do not enter the host by direct penetration of skin, there are five essential biological requirements for pathogenicity: colonize mucous surfaces; penetrate them; grow in the tissues; inhibit host defence; and damage the host. The cardinal fact about pathogenicity is that it is multifactorial. Many genes are involved. Their products, the determinants of pathogenicity (virulence determinants), are the molecular bases for the five essential biological properties (Smith 1995). The goal of studies on bacterial pathogens is to recognize these determinants, to identify them and to relate their structure to function.

In the past, most of our knowledge about these determinants has come from experiments with bacteria grown in culture. Almost all pathogens have been investigated and many determinants have been identified. Examples are the pili of gonococci, which aid mucosal colonization; the Ipa proteins of shigellae, which are involved in invasion of colonic epithelium; the enterochelin of *Escherichia coli*, which aids acquisition of iron for growth; the capsular polysaccharides of pneumococci that interfere with host defence; and the lethal toxin of diphtheria bacilli (Smith 1995; Finlay & Falkow 1997).

Now, the situation has changed. There is a burgeoning interest in the activities of bacteria within the infected host. Environmental conditions *in vivo* (osmolarity, pH, E_h, and nutrient and substrate availability) differ from those in laboratory cultures. They are more complex. Pathogens may be in films on mucosal surfaces or free in body fluids, cell cytoplasm or cell vacuoles; in all cases, the environment is neither simple nor defined. Also, the conditions change during the course of infection due to inflammation, tissue breakdown and spread from one anatomical site to another. Within 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 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 typhimuriun* 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

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 *et al*. 1997
	fluorescence-activated cell sorting (FACS)	rapid identification of host cells containing fluorescent bacteria	Valdivia & Falkow 1997*a*
	laser microprobe mass spectrometry	measuring viability of bacteria in biopsies by Na^+/K^+ ratios	Haas *et al*. 1993; Seydel *et al*. 1992
	photonic and radio detection of pathogens *in vivo*	pathogens made bioluminescent by a luciferase or radiolabelled by technetium-99 m	Contag *et al*. 1995; Perin *et al*. 1997
measuring enviromental parameters *in vivo*	quantitative fluorescence microscopy	measurement of fluorescing dyes that react to environmental factors	Akins *et al* 1995; Aranda *et al* 1992
	use of reporter genes	*LacZ* fusions to genes that respond to certain levels of compounds in the environment	Garcia-del Portillo *et al*. 1992; Pollack 1986
	X-ray microanalytical electron microscopy		Morgan 1985; Spencer *et al*. 1990
detection of genes expressed *in vivo*	*in vivo* expression technology (IVET)	genes expressed *in vivo* provide promoters for various reporting systems	Camilli *et al*. 1994; Camilli & Mekalanos 1995; Heithoff *et al*. 1997; Lowe *et al*. 1998; Mahan *et al*. 1994, 1995; Wang *et al*. 1996*a*,*b*; Young & Miller 1997
	differential fluorescence induction (DFI)	promoters of genes expressed *in vivo* drive expressions of green fluorescent protein	Valdivia & Falkow 1996, 1997*a*
	differential display of cDNAs	cDNAs prepared from mRNAs of genes expressed *in vivo* and compared with cDNAs from organisms *in vitro*	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 *et al*. 1995; Suk *et al*. 1995; Wallich *et al*. 1995
	labelling proteins with diaminopimelate	diaminopimelate used by bacteria and not by host cells	Burns-Keliher *et al*. 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 *et al*. 1995; Chiang & Mekalanos 1998; Coulter *et al*. 1998; Mei *et al*. 1997; Shea *et al*. 1996
	virulence complementation	complementation of avirulent strains by gene libraries from virulent strains	Collins 1996; Pascopella *et al*. 1994
global analysis of potential gene expression	complete genome sequencing	aids the identification of new genes expressed *in vivo*	Strauss & Falkow 1997; Tang & Holden 1999
	chip technologies	microarrays of probes allow monitoring of expression of many genes in parallel	De Saizieu *et al*. 1998; Lockhardt *et al*. 1996; Ramsay 1998; Schena *et al*. 1996

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 deficiences 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 metobolite 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 *Lac*Z 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 deficiences 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.

(i) *Confirming production* in vivo *and relevance to virulence of putative determinants recognized* in vitro

It is now standard practice in most studies of pathogenicity to confirm the production *in vivo* of putative determinants. Bacteria harvested directly from patients or infected animals can be examined by conventional methods (Smith 1990, 1996). The profiles of homogenates run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) can be examined for bands corresponding to those of purified putative determinants. If the latter are antigenic, specific antisera can be used to immunoblot the profiles and for fluorescence microscopy of bacteria in tissue sections. Also, convalescent sera from patients or animals can be examined for appropriate antibodies by using them to immunoblot SDS–PAGE profiles of *in vivo*- and *in vitro*-grown organisms. In addition, the new methods for detecting gene expression *in vivo* (table 1) can confirm production *in vivo* of putative determinants, e.g. *tcp* (the toxin-coregulated pilus) of *V. cholerae* by STM (Chiang & Mekalanos 1998). Usually the putative determinant is produced *in vivo* but not always. For example, capsular polysaccharide type 5 is formed by *Staphylococcus aureus in vitro* in aerated cultures but minimally in the lungs and nasal polyps of cystic fibrosis patients (Herbert *et al.* 1997). It is also usual to prove relevance to virulence of the putative determinant by showing that deficient mutants are less virulent than wild-type strains in animal models of infection, followed by complementing the deficient gene (Falkow 1988). In most cases, relevance to virulence is proven, but not always, even when the putative determinant is present *in vivo*. The 17 kDa product of the *ail* gene of *Yersinia enterocolitica* is formed in Peyer's patches of mice (Wachtel & Miller 1995) but experiments with an *ail*-deficient mutant indicated that the gene is not required, either for primary invasion or to establish systemic infection. In cell cultures, Opa proteins are determinants of cell invasion by gonococci grown *in vitro* (Dehio *et al.* 1998). Gonococcal strain FA1090 produces at least eight antigenically distinct Opa proteins. When an Opa-negative variant was inoculated into volunteers, Opa proteins were expressed in a large proportion of the re-isolates. The predominant Opa variants differed between subjects. Hence, Opa proteins are formed *in vivo*. However, one variant expressing Opa protein F, which was highly represented in the re-isolates, was no more infective for volunteers than an Opa-negative variant (Jerse *et al.* 1994).

Another encouraging trend is that results of cell culture tests for virulence determinants are being viewed in relation to the pathology of disease. In the forefront of such studies is penetration of the mucosa by intestinal pathogens for which the determinants of invasion of epithelial cell lines are known. However, *in vivo* these pathogens do not usually invade epithelial cells directly. They penetrate the mucosa via M cells present in Peyer's patches and elsewhere (Jepson & Clark 1998). Now, the determinants of invasion of cell lines are being investigated for a role in this process. Pioneering studies were done on *S. flexneri* (Sansonetti *et al.* 1999). Experiments with Hela cells showed that entry, intracellular movement and transfer between cells were determined by plasmid gene products IpaB, IpaC, IpaD and T_{CSD}. Then, experiments with confluent polarized colonic epithelial cell lines, e.g. CaCo-2 cells showed that shigellae could not penetrate the intact brush border that exists *in vivo* and is absent from Hela cells. *In vivo*, shigellae are ingested by M cells and delivered to macrophages in the *lamina propria*. IpaB causes apoptic death of the macrophages and IL-1β is released. Inflammation follows with disruption of the epithelial cells so that shigellae can invade the sides and bases of these cells, using the determinants recognized in the Hela cell studies. *In vivo*, *S. typhimurium* causes membrane ruffling of M cells, then is internalized leading to cell destruction as for tissue culture cells (Jones *et al.* 1994; Jepson & Clark 1998). Mutants deficient in salmonella pathogenicity island SPI1 genes, and thus unable to enter tissue culture cells, caused membrane ruffling and entered M cells but to a lesser degree than the wild-type (Clark *et al.* 1996; Jepson & Clark 1998). Also, they killed mice after oral inoculation but the LD_{50}s were

higher than for the wild-type. Hence, the determinants of invasion of cell lines by *S. typhimurium* have some role in invasion via the M-cell system *in vivo* but unknown determinants are also involved.

(ii) *Examining bacteria grown* in vivo *for hitherto unknown virulence determinants*

The fact that some virulence determinants may not be formed *in vitro* is generally accepted. Increasingly, the genes and their products in bacteria harvested from patients and infected animals are being compared with those of bacteria grown *in vitro* to reveal differences that may be biologically important. Also, for intracellular pathogens, organisms grown in macrophages are compared with those from cultures. Such studies are aimed at recognizing potential diagnostic aids and immunizing antigens as well as virulence determinants. Over the past ten years, conventional studies using SDS–PAGE have revealed many hitherto unknown bacterial components of numerous different pathogens (Smith 1990, 1996). Now, the new methods for detecting genes expressed *in vivo* (table 1) are recognizing new genes of potential importance.

Having recognized a previously unknown bacterial component, the next step is to prove that it is a virulence determinant by biological tests related to pathogenicity and virulence tests on appropriate mutants. Unfortunately, in conventional studies, this follow-up has not been as popular as the original demonstration of a new component. For example, in the intestinal lumen of mice, *Y. enterocolitica* produced a plasmid-encoded outer membrane protein (23 kDa), which had not been seen in culture. On invasion of Peyer's patches, this protein and two further proteins (210 and 240 kDA) were formed (Nauman *et al.* 1991). There the matter remains. The functions of these novel proteins in intestinal invasion have not been investigated. Follow-up on hitherto unknown genes revealed by the new methods has been much better. In many cases, virulence tests have been done on appropriate mutants and, for STM, the method itself detects only virulence genes. As a result, many new virulence genes have been recognized, e.g. by IVET and STM for *V. cholerae* (Camilli & Mekalanos 1995; Chiang & Mekalanos 1998), *S. typhimurium* (Hensel *et al.* 1995; Mahan *et al.* 1993), *Y. enterocolitica* (Young & Miller 1997) and *S. aureus* (Lowe *et al.* 1998; Mei *et al.* 1997).

(iii) *Identifying virulence determinants as infection proceeds, together with the regulatory processes involved*

There are some indications that the requirements for virulence determinants change as infection proceeds. For example, in experiments on gonococcal infection in volunteers (see § 4), there was an indication that gonococci lacking a sialylated lipopolysaccharide (LPS) are optimal for initial invasion of epithelial cells but that later, to cope with host-defence mechanisms, production of sialylated LPS is an advantage. However, proof of specific changes in virulence-determinant complements at different stages of infection in animals has not been obtained. In studies with macrophages, switch-on of genes at different times during an infection has been detected. Differential fluorescence induction (DFI) (table 1) distinguished two classes of macrophage-induced genes, one induced within an hour of *S. typhimurium* entering and the other after four hours (Valdivia & Falkow 1997*b*).

Some observations have been made on the operation *in vivo* of regulatory systems detected *in vitro*. Two examples are discussed. The ToxR/ToxS virulence regulon of *V. cholerae* comprises over 20 genes involved in colonization (e.g. *tcp* genes which code for toxin-coregulated pili) and production of cholera toxin (Skorupski & Taylor 1997; DiRita *et al.*, this issue). It depends on a transcriptional activator ToxR, ToxS (a stabilizer of ToxR) and ToxT, another transcriptional activator that is positively regulated by ToxR. The regulon is modulated *in vitro* by temperature, osmolarity, pH, oxygen status and availability of amino acids. The PhoP/PhoQ regulator of *S. typhimurium* is a two-component regulatory system. PhoQ is the sensor and PhoP is the transcriptional activator that is phosphorylated by PhoQ. It controls expression of more than 40 genes, including those needed to resist killing within phagocytes and those involved in lipid A synthesis (Garcia Vescovi *et al.* 1994; Guo *et al.* 1997). *In vitro*, it is affected by pH, oxygen tension, carbon and nitrogen starvation and phosphate and magnesium concentrations (Garcia Vescovi *et al.* 1996).

In considering whether these and other regulons operate *in vivo*, it should be remembered that production *in vivo* of a virulence determinant whose formation *in vitro* is controlled by a certain regulator does not necessarily mean that control *in vivo* is effected by the same regulator. To confirm that the regulator is involved, expression of its genes should be detected *in vivo*; deletion of the genes should reduce virulence; and relevant environmental parameters should be present at the level at which they are effective *in vivo*.

The IVET method did not detect expression of the cholera toxin gene nor other ToxR/ToxS regulated genes in the intestines of infected mice (Camilli & Mekalanos 1995); but STM in the same model detected mutants with insertions in *tcp* and *toxT* (Chiang & Mekalanos 1995). IVET showed that the genes of the PhoP/PhoQ system were expressed in mice infected with *S. typhimurium* (Heithoff *et al.* 1997; Conner *et al.* 1998). However, in infected macrophages DFI and IVET identified two and one gene, respectively, which were not controlled by PhoP/PhoQ (Valdivia & Falkow 1997*a*; Heithoff *et al.* 1999), thus indicating that this system is not the only one operating. With regard to virulence tests on mutants, a ToxR/ToxS-deficient mutant of *V. cholerae* produced less colonization and diarrhoea in volunteers than did the wild-type (Herrington *et al.* 1988), and PhoP/PhoQ-deficient mutants were avirulent for mice (Miller *et al.* 1989; Garcia Vescovi *et al.* 1996). The influence of environmental parameters is discussed later.

Recently, two new regulatory systems have been described, quorum sensing and type III secretion (Williams *et al.*, this issue; Cornelis, this issue). They are summarized here, in order to ask some questions about their operation *in vivo*. In quorum sensing, transcriptional activators of virulence-determinant production only go into operation when a significant cell population has been attained (Guangyong *et al.* 1995; Winson *et al.* 1995). The cell-density dependency reflects a cell-to-cell communication system based on accumulation of signal molecules to

a threshold concentration. The signalling molecules for *P. aeruginosa* are N-(3-oxododecanoyl)-L-homoserine lactone and N-butanoyl-L-homoserine lactone, which switch on two transcriptional activators, LasR (regulates expression of the elastase LasB) and RhiR (regulates expression of rhamnolipid), respectively (Lafiti *et al.* 1996; Pesci & Iglewski 1997). Together they regulate virulence determinants, secondary metabolites and survival in the stationary phase. The signalling molecule for *S. aureus* is an octapeptide which activates the accessary gene regulator (Agr), which positively regulates production of extracellular toxins (e.g. α-toxin, β-toxin and toxic shock syndrome toxin 1) and fibronectin-binding proteins (Guangyong *et al.* 1995).

In vivo, quorum sensing cannot operate early in infection because the numbers of pathogens are too small. To be certain that it operates later, evidence is needed for expression of relevant genes, reduced virulence if these genes are mutated, attainment of requisite population densities and detection of signalling molecules. Some of this evidence is emerging. Examination of RNAs from *P. aeruginosa* in the sputum of cystic fibrosis patients indicated that *lasR* transcription occurs and may coordinately regulate virulence genes *lasA*, *lasB* and *toxA* (Storey *et al.* 1988). Also, *lasR*-deficient mutants of *P. aeruginosa* were less able than the wild-type to produce pneumonia in neonatal mice (Tang *et al.* 1996). They were, however, equally able to infect the corneas of mice (Preston *et al.* 1997). IVET demonstrated the expression of the *agrA* gene of *S. aureus* in mice (Lowe *et al.* 1998) and staphylococcal mutants defective in *agrA* were of reduced virulence (Gillaspy *et al.* 1995).

Type III secretion systems respond when bacteria contact eukaryotic cells. They induce secretion of virulence determinants from the bacterial cell and deliver them into host cells (Hueck 1998). A good example is the Yop protein system of *Yersinia* spp. (Cornelis 1998; Cornelis *et al.* 1998). The Yop virulon is encoded by a 70 kb plasmid, pYV. It consists of (i) a secretion apparatus Ysc comprising over 20 proteins, (ii) a delivery (to host cells) system consisting of YopB, YopD, LcrV and YopQ/YopK, (iii) a control element, YopN, TycA and LcrG, and (iv) a set of effector proteins that harm phagocytes, YopE, YopH, YpkA/YopO, YopM and YopT. Transcription of genes is influenced by temperature changes and cell contact.

S. typhimurium has two type III secretion systems comprising many genes dealing with secretion, delivery, regulation and production of effects on host cells. One deals with export and translocation to host cells of invasion proteins that are responsible for membrane ruffling and entry of epithelial cells in culture (Galan 1996; Hueck 1998). The other is involved with virulence in mice and proliferation in macrophages (Shea *et al.* 1996; Hensel *et al.* 1998).

In proving operation *in vivo* of type III secretion systems, it will not be easy to show that they are switched on by contact with relevant host cells in tissue sections or biopsies as has been demonstrated for contact with tissue culture cells (Petterson *et al.* 1996). However, the new methods such as CLSM may help in this respect. It will be easier to demonstrate the expression of the regulatory genes of these complex systems *in vivo* and reduction of virulence when these genes are mutated. Indeed, this has been done for the second type III secretion system of *S. typhimurium*. The system was discovered by STM showing expression of relevant genes *in vivo*. Mutation of one regulatory gene, P_3F_4, resulted in loss of virulence (Shea *et al.* 1996).

(b) *Host factors*

First, as for growth, factors in the environment that could affect production of virulence determinants (osmolarity, pH, E_h and metabolites) should be recognized and their levels or concentrations *in vivo* determined. Then, attempts should be made to mimic *in vitro* the conditions *in vivo* and observe the effect on virulence-determinant production and its regulation. Although receiving far less attention than bacterial activities, such experiments are beginning. Six invasion genes of *S. typhimurium* were maximally expressed *in vitro* at an oxygen tension, osmolarity and pH likely to exist in the ileum (Bajaj *et al.* 1996). Conditions designed to mimic those of the intestine showed that two type III secretion genes of *S. typhi*, *invG* and *prgH* were induced by high osmolarity, anaerobic conditions and pH 6.5, and strongly repressed at pH 5.0 (Leclerc *et al.* 1998). When *S. typhi* was grown at an osmolarity (300 mM NaCl) similar to that of the human intestine, production of flagellin and salmonella invasion proteins increased, and that of the surface Vi antigen, which prevents their secretion, was depressed (Arricau *et al.* 1998). This would facilitate contact with and entry into epithelial cells. The position was reversed at an osmolarity (150 mM NaCl) similar to that within the tissues (Arricau *et al.* 1998). The *Yst* regulated toxin of *Y. enterocolitica* is produced at 37 °C *in vivo* but not in culture media unless the temperature is below 30 °C. However, if the osmolarity and pH of the medium are adjusted to values normally present in the ileum, toxin production at 37 °C occurs (Mikulskis *et al.* 1994). Similarly, *in vitro* expression of the invasin gene *inv* by *Y. enterocolitica* is depressed at 37 °C compared with that at lower temperatures, but it increases significantly if the pH is less than 7 (which occurs in the stomach) and when concentrations of Na^+ increase (which occurs near the enterocyte brush border) (Pepe *et al.* 1994). The level of *inv* expression in the mouse intestine is comparable to that at 23 °C *in vitro*.

In some cases the environmental conditions that affect regulons *in vitro* have been shown to exist *in vivo*. The PhoP/PhoQ system of *S. typhimurium* appears to be controlled *in vivo* by Mg^{2+} as it is *in vitro*. During infection, *S. typhimurium* resides in phagosomes where the Mg^{2+} concentration (estimated 50–100 μM) is permissive for *phoP/phoQ* expression, unlike the high concentrations (0.5–1.0 mM) found in cytosols and body fluids (Garcia Vescovi *et al.* 1996). Also, a *phoQ* mutant that was less responsive to Mg^{2+} was of attenuated virulence for mice (Garcia Vescovi *et al.* 1996). In contrast, the position on the ToxR/ToxS system is not as clear. Classic strains of *V. cholerae* form toxin maximally *in vitro* at low temperatures and under aerobic conditions at low pH, whereas in the human intestine, toxin production occurs at 37 °C and under anaerobic conditions at high pH. The actual environmental control of the ToxR/ToxS system *in vivo* is still under investigation (Skorupski *et al.* 1997; DiRita *et al.*, this issue).

4. SIALYLATION OF GONOCOCCAL LPS BY HOST CMP-NANA AND EFFECT OF LACTATE: A PARADIGM FOR INVESTIGATION OF BEHAVIOUR *IN VIVO*

Sialylation of gonococcal LPS by host-derived cytidine 5′-monophospho-N-acetyl neuraminic acid (CMP-NANA) and lactate has a major influence on many aspects of gonococcal pathogenicity. This was revealed by investigating the cause of a biological property of gonococci in urethral exudates which was lost on subculture *in vitro*. This work shows how bacterial activities *in vivo*, relevant host factors and the metabolism concerned can be identified. References to papers up to 1995 are given in Smith *et al*. (1995).

(a) *Sialylation of LPS by host CMP-NANA affects pathogenicity*

Gonococci in urethral exudates are resistant to complement-mediated killing by fresh human serum. In most cases, resistance is lost on one subculture *in vitro* but it can be restored by incubation with blood cell extracts. Fractionation showed that the resistance inducing activity is due to CMP-NANA. After growing gonococci with CMP-^{14}CNANA, autoradiography of LPS bands separated by SDS–PAGE showed that some, but not all, LPS components are sialylated. One sialylated component of 4.5 kDa is conserved in many strains and its side chain is Galβ1-4GlcNacβ1-Galβ1-4Glc. The sialylated LPS forms an irregular surface coat, which is seen on gonococci in urethral exudates whose LPS was shown to be sialylated. A previously unknown gonococcal sialyltransferase was demonstrated in gonococcal extracts. LPS sialylation is responsible for serum resistance since conversion to resistance accompanies sialylation by CMP-NANA and reversion to sensitivity occurs when sialyl groups are removed by neuraminidase. The crucial importance of LPS sialylation in serum resistance of gonococci is now generally accepted (Vogel & Frosch 1999). Sialylation of LPS also interferes with the following host-defence mechanisms: absorption of complement component C3; ingestion and killing by PMN phagocytes; killing by antisera against gonococcal proteins; and stimulation of the immune response. On the other hand, sialylation prevents invasion of epithelial cells. Observations on volunteers were consistent with these results. When they were inoculated with a strain whose LPS could not be sialylated, a variant was selected *in vivo* whose LPS could be sialylated (Schneider *et al*. 1991). This variant, recovered from the volunteers, was more virulent than the parent strain provided it was inoculated after being grown in a medium without CMP-NANA (i.e. its LPS was not sialylated so that it could invade epithelial cells) (Schneider *et al*. 1995, 1996). If the inoculum was grown with CMP-NANA, it did not infect volunteers so well, consistent with inhibition by LPS sialylation of the ability to invade epithelial cells (Schneider *et al*. 1996).

A sialyltransferase-deficient mutant, in contrast to its parent strain, did not become serum resistant when incubated with either CMP-NANA or blood cell extracts (Bramley *et al*. 1996). Hence, the latter do not contain a mechanism for sialylating gonococcal LPS, which is independent of CMP-NANA. Also, unlike the wild-type, incubation of the mutant with CMP-NANA did not increase resistance to ingestion and killing by PMN phagocytes, killing by antiserum to porin I and human complement, binding of C3 of complement, and invasion of epithelial cells (Gill *et al*. 1996). The mutant was unable to sialylate any of its LPS components, which were shown by mass spectrometry to be similar to those of the parent strain. They included components sialylatable by the sialyltransferase from the parent strain, such as the 4.5 kDa conserved component mentioned above (Crooke *et al*. 1998). Hence, loss of ability of the mutant to be converted by CMP-NANA to resistance to serum killing, and all the other facets of pathogenicity mentioned above, is attributed to loss of sialyltransferase activity rather than inability to synthesize the LPS substrate for sialylation. Final confirmation would have come from complementation of the mutant by the gene for the sialyltransferase which has been cloned and sequenced (Gilbert *et al*. 1996). This could not be achieved (Crooke *et al*. 1998) so it seems that multiple genetic loci may be essential for LPS sialylation. The mutant has not been examined in human volunteers. A sialyltransferase-negative mutant of gonococcal strain FA 1090 was as infective as the wild-type for volunteers (Cannon *et al*. 1998), but this is not unexpected because this strain is fully resistant to serum killing (Cohen *et al*. 1994) and does not require LPS sialylation to make it so.

(b) *Host lactate enhances LPS sialylation, and LPS and protein synthesis*

Another blood cell factor, which enhances the ability of CMP-NANA to sialylate gonococcal LPS and to induce serum resistance, has been identified as lactate (Parsons *et al*. 1996*a*). The enhancement occurs with minute quantities of lactate in a defined medium containing high concentrations of glucose. The action of lactate is separate from that of CMP-NANA because enhanced sialylation occurred when gonococci were pretreated with lactate (Parsons *et al*. 1996*b*). Lactate did not increase the gonococcal content of sialyltransferase (Gao *et al*. 1998). On the other hand, there was a marked increase in LPS synthesis (10–20%), which could explain the enhancement of sialylation because additional receptors for sialyl groups are provided. The increase in LPS synthesis was paralleled by increases in protein synthesis and ribose content, presumably reflecting additional ribosome production (Gao *et al*. 1998).

(c) *Metabolic effects of lactate on gonococci growing in a medium containing glucose, as occurs* in vivo

The increases in LPS, protein and ribose synthesis, first noticed under the conditions for detecting enhancement of sialylation by lactate, also occurred when both glucose and lactate concentrations in the defined medium were adjusted to levels akin to those occurring *in vivo* (Gao *et al*. 1998). Hence, there appears to be a general stimulation of gonococcal metabolism when lactate is added to media containing glucose, and there is other evidence. Lactate increased oxygen consumption by gonococci in a solution containing glucose (Britigan *et al*. 1988). Also, in the above-defined medium, growth rate was faster and lactate was metabolized side-by-side with glucose and more rapidly (Regan *et al*. 1999). When gonococci were grown with ^{14}C-labelled lactate in this medium (Yates *et al*. 1999), tricene SDS–PAGE on homogenates showed

that lactate is not a general carbon source. Label was concentrated in a low M_r component, LPS and a few proteins. N-terminal sequencing of the three most heavily labelled proteins showed one (M_r *ca.* 58 kDa) to be the chaperone, GroEl and another (M_r *ca.* 35 kDa) porin 1B. Nuclear magnetic resonance after ^{13}C labelling, and thin layer chromatography following ^{14}C labelling (Yates *et al.* 1999), showed the low M_r component to be lipid. Gonococcal membrane lipids consist mainly of phosphatidyl ethanolamine and glycerol esterified to palmitic, myristic, a 16:1 and a 18:1 acid (Sud & Feingold 1975). In the glucose-containing medium, the carbon atoms from the ^{13}C lactate were incorporated specifically into the fatty acid portions, in contrast to both glycerol and fatty acid moeities when ^{13}C glucose was used without lactate present. The location in the LPS of the ^{14}C label from the lactate is not yet known but the fatty acid residues seem likely.

The incorporation of lactate carbon into GroEl is interesting in two respects. GroEl would ensure correct folding of the products of the large increase in protein synthesis (Gao *et al.* 1998). Also, it could contribute to the inflammation seen in gonorrhoea, since it is a potent stimulator of relevant cytokines (Coates & Henderson 1998); and it is significant that patients have high levels of antibody to GroEl (Demarco de Hormaeche *et al.* 1991). Porin 1B plays a major metabolic role, membrane transport, in gonococci (Gotschlich *et al.* 1987) and can contribute to pathogenicity by inserting into host-cell membranes (Bjerknes *et al.* 1995). Stimulation of lipid formation would aid membrane synthesis and therefore metabolism and growth. It is fascinating that gonococci have adapted to use lactate and glucose, which are ubiquitous *in vivo,* to produce a vibrant metabolism and a large content of virulence determinants such as LPS and GroEl. The marked effects of minute amounts of lactate on LPS, protein and ribose synthesis in a medium containing large quantities of glucose suggests that lactate may have a signalling as well as a metabolic role.

(d) *Extension of the work to meningococci*

The work on gonococci stimulated similar investigations on meningococci. Some strains contain LPS components that are endogenously sialylated (serogroups B, C, W and Y) and others have components that can be sialylated exogenously by host CMP-NANA (groups A and 29E) (Smith *et al.* 1995). An LPS sialyltransferase is present (Smith *et al.* 1995). LPS sialylation affects facets of pathogenicity but not as markedly as for gonococci because capsular polysaccharide is the more powerful virulence determinant. It is sometimes difficult to distinguish between their respective roles. LPS sialylation inhibits meningococcal invasion of epithelial cell lines, endothelial cells and mono- and PMN phagocytes (McNeil & Virgi 1997; Virgi *et al.* 1993). Also, it interferes with opsonophagocytosis of some strains (Smith *et al.* 1995). The position regarding serum resistance is equivocal; some papers indicate that LPS sialylation is important (Esterbrook *et al.* 1997; Kahler *et al.* 1998) and others that it is less so (Vogel *et al.* 1997; Vogel & Frosch 1999). In an outbreak of group B meningitis, an immunotype capable of LPS sialylation was associated with invasive disease and an immunotype incapable of LPS sialylation with the carrier state (Smith *et al.* 1995). The effect of lactate on meningococci has not yet been investigated.

5. CONCLUDING REMARKS

I hope this paper has made clear the pertinent questions about the behaviour of bacterial pathogens *in vivo*; and has indicated how they might be answered, despite difficulties in some areas, by a combination of conventional and newly devised methods.

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