

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 (SDSPAGE) 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 SDSPAGE 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 apoptotic death of the macrophages and IL-1 β 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 LD₅₀s 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 1997b).

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 volume). 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 1997a; 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 RhlR (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 accessory 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 (Pettersen *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, P₃F₄, 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 Mg²⁺ as it is *in vitro*. During infection, *S. typhimurium* resides in phagosomes where the Mg²⁺ 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 Mg²⁺ 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 volume).

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-¹⁴CNANA, 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 Gal β 1-4GlcNac β 1-Gal β 1-4Glc. 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