Abstract

*Bordetella pertussis* and *Bordetella bronchiseptica* are respiratory pathogens of humans and animals respectively. Unlike many bacteria, they are able to efficiently colonise healthy ciliated respiratory mucosa. This characteristic of *Bordetella* spp. can potentially be exploited to develop efficient live vaccines and vectors for delivery of heterologous antigens to the respiratory tract. Here we review the progress in this area.

Keywords: Bordetella; Mucosal vaccine; Heterologous antigen delivery

1. Mucosal immune system and mucosal vaccines

Most infectious diseases are initiated when a pathogen makes contact with one of the exposed surfaces of the host. Commonly this is the mucous membranes lining the respiratory or gastrointestinal tracts. Protection of mucosal surfaces is mediated by both non-specific (innate) and specific (acquired) defence systems. The acquired mucosal immune system is comprised of specialised tissues and organs that are responsible for capture, uptake and processing of immunogens and the induction of effector functions. The innate and acquired defences of the mucous membranes provide a first line barrier to infection from the majority of pathogens. Pre-arming of the specific immune system against particular pathogens can be achieved by immunisation.

In the majority of cases, natural infection usually results in strong and long-lasting immunity. Infection stimulates the local immune system at the site of infection and systemic immune responses, even if the organism does not penetrate mucosal epithelial cells. Cell-mediated (CMI) and humoral immune responses are both effectively elicited by infection with most pathogens. Consequently, immunity resulting from infection with the wild-type pathogen is often superior to that achieved by vaccination, particularly if CMI or responses to multiple antigens are needed for protective immunity. Live vaccines that are able to cause self-limiting, clinically inapparent infections therefore usually come closest to achieving the level of immunity resulting from natural infection. This may be for a number of reasons including: the stimulation of the appropriate immune responses; persistence of the organism (increased antigenic load); colonisation of particular cells or tissues, e.g., antigen presenting cells; preferential expression of particular antigens in vivo.

Parenteral (traditional) immunisation alone is extremely poor at initiating mucosal immune responses [1,2]. Also the re-use of needles is still common in the developing world and thus parenteral immunisation carries the risk of transmitting blood-borne infections such as HIV and hepatitis B. It would therefore be advantageous to administer vaccines mucosally even if mucosal immunity does not contribute to protection against a disease, e.g., tetanus. Direct contact of the immunogen with the appropriate mucosal surface is the most effective means of eliciting a local secretory immune response. Unfortunately, most non-viable, and particularly soluble antigens are very poor immunogens when given by a mucosal route.

One of the major goals of modern vaccinology is to devise means of improving the immunogenicity of mucosally delivered vaccines. Several approaches are being in-
vestigated including non-living systems [3,4]. Another approach is to express the gene encoding the antigen in an attenuated bacterium which serves to deliver the antigen to the immune system [5,6]. A prerequisite for developing such vaccines are safe, effective and, preferably, well-defined attenuated bacterial vaccine strains. The majority of the work using bacteria as live vectors has so far focused on attenuated strains of *Salmonella* spp. [6,7]. Oral immunisation with recombinant *Salmonella* strains can stimulate CMI, cytotoxic T-cells and circulating and secretory antibodies to the heterologous antigen [6,7]. Attenuated *Salmonella* spp. have also been used to deliver DNA vaccines into eukaryotic cells [8]. More significantly, protective immunity to challenge with heterologous organisms or their products has also been demonstrated in many cases [6].

### 2. The *Bordetellae*

The *Bordetellae* are a group of highly related small Gram-negative bacterial pathogens of mammals and birds. The most extensively studied of these are the respiratory pathogens *B. pertussis* and *B. bronchiseptica*. *B. pertussis* naturally only infects humans and is the causative agent of whooping cough in infants and persistent respiratory infections in adults. *B. bronchiseptica*, in contrast, is able to colonise the respiratory tract of a large number of different animals and causes respiratory infections in farm, companion and wild animals [9]. In addition *B. bronchiseptica* is occasionally isolated from the respiratory tract of humans, although these individuals tend to be immunocompromised [10]. *Bordetella* spp. produce an array of virulence factors the expression of which is generally controlled by the two-component regulator BvgAS, in response to environmental stimuli [11,12]. *B. pertussis* and *B. bronchiseptica* are believed to enter the respiratory tract in aerosol droplets generated by the coughing of an infected individual. Unlike most bacterial respiratory pathogens, *B. pertussis* and *B. bronchiseptica* are able to adhere efficiently to healthy, rapidly beating ciliated epithelial cells. Here the organisms proliferate and produce factors that counteract host defences and this prevents their elimination. *B. pertussis* and *B. bronchiseptica* do not normally invade beyond the respiratory tract although it appears that they are able to survive intracellularly in a number of cell types including professional phagocytes [11]. As the respiratory tract becomes impaired infected individuals are then particularly susceptible to secondary infections. Vaccination has proven effective for preventing infection of humans and animals by *Bordetella* spp. Killed whole cell and subunit vaccines have been used to immunise parenterally to protect humans against *B. pertussis* and mammals against *B. bronchiseptica*. It is apparent that both local secretory antibodies and CMI are essential for full immunity to *B. pertussis* and *B. bronchiseptica* [11,13]. The presence of a number of *Bordetella* antigens in vaccine preparations seems to be crucial for stimulating protective immunity and these include pertactin, filamentous haemagglutinin (FHA) and fimbriae (and pertussis toxoid in the case of *B. pertussis*) [11].

A number of characteristics of *B. bronchiseptica* and *B. pertussis* make them attractive candidates for developing efficient live respiratory vaccines and antigen delivery systems. As a consequence of their efficient colonisation of the respiratory tract they have a very low infectious dose. They generally do not cause systemic infections. Their genetics are advanced and they are relatively simple to genetically manipulate. Relatively good animal models exist to evaluate candidate vaccine strains (particularly for *B. bronchiseptica*). *Bordetella* spp. also possess all of the known bacterial protein secretion systems, so it should be feasible to secrete all types of foreign antigens if required. This review will describe the development of attenuated strains of *B. pertussis* and *B. bronchiseptica* and their use as live mucosal vaccines and as vectors for respiratory delivery of foreign antigens.

### 3. Attenuated *Bordetella* strains as live vaccines

#### 3.1. *B. bronchiseptica*

Attenuated strains of *B. bronchiseptica* have been shown to function as safe and effective live intranasal (i/n) vaccines in mice, guinea pigs, pigs, dogs and cats and commercial vaccines exist for the latter three species [14–23]. In certain cases experimental details of the effectiveness of the commercial vaccines have not been published. Also details of the vaccine strains are scant [24]. None of the commercial vaccine strains are known to be genetically modified. Since the basis of attenuation is unknown, there is the potential that the strains may revert to full or partial virulence. Also the attenuating lesion may affect expression of heterologous antigens. Nevertheless, the success of

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**Table 1**

Comparison of *B. bronchiseptica* and *B. pertussis*

<table>
<thead>
<tr>
<th></th>
<th><em>B. bronchiseptica</em></th>
<th><em>B. pertussis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Host range</strong></td>
<td>Wide</td>
<td>Narrow</td>
</tr>
<tr>
<td><strong>Murine model</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>– Natural pathogen</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>– Infective dose</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>– Animal-to-animal transmission</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>– Serum antibody response after infection</td>
<td>Rapid</td>
<td>Delayed</td>
</tr>
<tr>
<td><strong>Live vaccine in clinical use</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Culture in vitro</strong></td>
<td>Easy</td>
<td>Problematic</td>
</tr>
<tr>
<td><strong>Growth rate in vitro</strong></td>
<td>Rapid</td>
<td>Slow</td>
</tr>
<tr>
<td><strong>Survival in environment</strong></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td><strong>Size of genome (Mb)</strong></td>
<td>5.3</td>
<td>4.1</td>
</tr>
<tr>
<td><strong>Estimated number of genes</strong></td>
<td>~4900</td>
<td>~3800</td>
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*Julian Parkhill, Sanger Centre, Cambridge, personal communication.*

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live i/n *B. bronchiseptica* vaccines in a variety of different species demonstrates the validity of this approach.

The following describes live vaccination with *B. bronchiseptica* strains for which there is detailed information. A dermonecrotic toxin (DNT) mutant of *B. bronchiseptica* was isolated after serial passage in vitro [23]. The DNT- strain did not produce adverse effects following i/n or intramuscular vaccination of guinea pigs [23]. Animals vaccinated by either route developed high-titre serum antibodies against *B. bronchiseptica* and were protected from challenge with wild-type organisms [23].

A temperature-sensitive urease (3) strain of *B. bronchiseptica* was isolated after mutagenesis with nitrosoguanidine and UV irradiation [16]. The mutant was unable to grow above 34°C and following i/n inoculation of guinea pigs the organism was isolated in moderate numbers from the nasal turbinates but not the lungs. i/n immunization of guinea pigs with the mutant conferred high-level protection against lethal challenge with a wild-type strain [16]. Interestingly, although the immunised animals developed serum anti-*B. bronchiseptica* antibodies the levels were not very high suggesting other mechanisms of protection. In support of this, nasal IgA anti-*B. bronchiseptica* titres in dogs immunised i/n with a live *B. bronchiseptica* strain were found to correlate with protection from clinical disease following challenge with virulent *B. bronchiseptica* [15].

### 3.2. B. pertussis

There are very few reports on the use of naturally occurring attenuated strains as live vaccines. An atypical strain that was isolated from the urine of a child with pertussis was used as a live vaccine in mice [25]. Mice that were immunised orally with this strain exhibited greater protection against intracerebral challenge with *B. pertussis* than mice that received the vaccine intraperitoneally [25]. A pertussis toxin (PTX)-deficient strain of *B. pertussis* has been used as a live vaccine and vector in mice (see below).

### 3.3. Genetically defined attenuated live **Bordetella** vaccine strains

Molecular biology techniques now allow precise changes to be made in specific genes to create stable genetically defined attenuated bacterial mutants. Different classes of genes can be targeted to create an attenuated strain. One possibility is to knock out a gene that encodes an important virulence determinant, such as a toxin or an adhesin. This may alter the strain’s ability to colonise or to survive in vivo, or make it less toxic to the host (see below). Another approach to attenuating a bacterial pathogen is to knock out gene(s), which are involved in a metabolic pathway whose function is crucial to in vivo survival and growth. Such mutations affecting the aromatic (pre-chorismate) pathway (*aro* mutants) have been intensively studied [26,27]. Aro- strains of both Gram-negative and Gram-positive bacterial pathogens are highly attenuated [6,28–33]. Blocking the pre-chorismate pathway prevents the formation of aromatic amino acids, p-aminobenzoic acid and other aromatic compounds. There are a number of advantages to attenuation by inactivating a metabolic pathway gene. For example, the candidate vaccine strain will still express all the virulence determinants, which are
often also the critical targets for stimulating protective immune responses. This is particularly pertinent for *Bordetella* spp. where protection is dependent on immune responses to a number of virulence factors [11].

*B. pertussis aroA* mutants are highly attenuated for lung colonisation of mice. In mice immunised with this strain no bacteria could be detected in the lungs after 8 days [34]. Mice given a single dose of a *B. pertussis aroA* strain by aerosol and then subsequently challenged with a wild-type *B. pertussis* strain cleared the challenged strain from their lungs more efficiently than control mice (unpublished observation). Mice that received three doses of the *B. pertussis aroA* strain over the course of 7 days exhibited much higher levels of protection. Following challenge, the wild-type strain was only present in the lungs of the immunised mice on the day after challenge (and only in one of four mice) [34]. In contrast, 18 days after challenge the wild-type strain was still present in three of four mice. Interestingly, only very low levels of serum anti-*B. pertussis* antibodies were present in the sera of immunised mice prior to challenge although the titre rose rapidly after challenge (cf. *B. bronchiseptica aroA* mutant below). The serum anti-*B. pertussis* antibody response of the naive control group (i.e. mimicking a natural infection) was also notable. High-titre antibodies were only detected in the sera ~25 days after *B. pertussis* was cleared from the lungs. This has also been reported in other studies of *B. pertussis* infection in mice [13,35].

It is important to note that *B. pertussis* only naturally infects humans, and high doses of *B. pertussis* are needed to establish infection in the murine respiratory tract. *B. pertussis* is usually cleared from the respiratory tract within 4–5 weeks after infection. Also animal-to-animal transmission of *B. pertussis* between mice does not occur. This is in contrast with *B. bronchiseptica*, which naturally causes respiratory infection in mice and other small mammals. *B. bronchiseptica* can establish infection in mice from a relatively low infectious dose, infection can spread to other mice and *B. bronchiseptica* can persist in the respiratory tract for longer than *B. pertussis*. Table 1 compares characteristics of *B. bronchiseptica* and *B. pertussis*. Despite *B. bronchiseptica* being able to colonise the respiratory tract more efficiently than *B. pertussis*, a *B. bronchiseptica aroA* mutant was as highly attenuated for lung colonisation in mice as its *B. pertussis aroA* counterpart (Fig. 1) [34,36]. However, the immune response of mice i/n immunised with a *B. bronchiseptica aroA* strain differed considerably from that of mice immunised with the *B. pertussis aroA* strain. Mice were immunised i/n with the *B. bronchiseptica aroA* strain with a single priming dose (1×prime) or with a three-dose prime (3×prime) and both groups received a single-dose boost 36 days later (note the *B. bronchiseptica aroA* strain was also expressing a foreign antigen, fragment C of tetanus toxin, see below). Significant anti-*B. bronchiseptica* serum antibody responses were detected before boosting even in the mice that received a single dose of vaccine, contrasting sharply with what was observed in *B. pertussis* [34,36]. This antibody response was directed against a variety of polypeptides (Fig. 2) and also lipopolysaccharide (unpublished observations).

Upon challenge, immunised mice exhibited a significantly enhanced clearance of *B. bronchiseptica* from their respiratory tract compared to control mice. The effect of immunisation on the clearance of *B. bronchiseptica* was most pronounced in the lungs and the effect was least in the nasal cavity [36]. The challenge strain was still present in all regions of the respiratory tract of control mice 56 days after challenge. Interestingly, in both immunised and control animals *B. bronchiseptica* did not clear fully from the nasal cavity despite the presence of high titre serum and nasal (IgA) anti-*B. bronchiseptica* antibodies [36].

### 3.4. *B. pertussis* as a vector for heterologous antigens

All of the studies on *B. pertussis* as a carrier have been carried out by Camille Locht and co-workers. Their initial studies looked at using *B. pertussis* to deliver the *Schistosoma mansoni* glutathione-S-transferase (Sm28GST) to mice [35]. The Sm28GST gene was introduced into the *B. pertussis* chromosome by insertion into the *fhaB* gene, which encodes FHA. FHA, an important protective antigen, is a major surface-associated and secreted protein of *B. pertussis* and it also appears to have immunomodulatory properties [37,38]. The fusion to FHA allows surface presentation and secretion of the fused passenger antigen. Expression of polypeptides using this approach may be of particular use for antigens which are naturally secreted and recognised by the immune system in this context.

The Sm28GST/FHA fusion was detected both on the surface of *B. pertussis* cells and in the extracellular environment. When compared to a wild-type *B. pertussis* background, the recombinant strain was equally able to colonise the respiratory tract of mice [35]. Four weeks after immunisation with this strain anti-Sm28GST and anti-FHA IgA and IgG could be detected in mouse bronchoalveolar lavage fluid (BALF). Despite this, serum anti-Sm28GST IgA and IgG could not be detected. Serum anti-FHA IgA and IgG antibodies were detected 40–50 days after immunisation [35]. In boosting of immunised mice with recombinant Sm28GST resulted in a significant anti-Sm28GST IgG serum response and increased anti-Sm28GST IgA titres in BALF [39]. Following challenge with *S. mansoni*, mice that received *B. pertussis* Sm28GST/FHA followed by recombinant Sm28GST exhibited a 33% reduction in worm burden and a 43.5% reduction in eggs recovered [39]. This was greater than in mice that received *B. pertussis* Sm28GST/FHA alone [39].

A *B. pertussis* mutant unable to produce PTX has also been studied as a carrier for FHA–antigen fusions. In mice
immunised with a PTX—strain the anti-FHA antibody response was initiated earlier and the final anti-FHA titres were higher than in mice infected with the wild-type parent [40]. The ptx operon in the B. pertussis strain Sm28GST/ FHA was deleted to create strain BPNX. Unlike its parent strain, a single i/n immunisation of mice with BPNX elicited serum anti-Sm28GST antibodies. Following S. mansoni challenge, BPNX-immunised mice exhibited a 56% reduction in worm counts and a 60% reduction in egg deposition in the liver. This level of egg reduction is reported to reduce pathology from S. mansoni infection to insignificant levels [40].

The results indicate that the delayed serum antibody to B. pertussis seen in B. pertussis-infected mice is possibly mediated by PTX (N.B., B. bronchiseptica does not produce PTX) and using B. pertussis ptx—carrier strains may be a general means of enhancing the serum antibody response to heterologous antigens. However, as described below, this has not proved to be the case for a number of antigens.

The Neisseria meningitidis group B transferrin binding protein B (TbpB) has been studied as a candidate antigen for an anti-N. meningitidis vaccine. Attenuated B. pertussis have been used as a delivery vehicle for this antigen [41]. In this case the TbpB protein and fragments of the protein were fused to a truncated version of FHA (Fha44) that contains the N-terminal secretion signals. The reason for this is that Fha44 was found to be secreted by B. pertussis more efficiently than full-length FHA [41]. TbpB fused to FHA was expressed in vitro but was found to be unstable on multicopy plasmids in B. pertussis in vivo [41]. To overcome this the genes for the fusions were integrated into the chromosome of B. pertussis [41]. Mice immunised i/n with TbpB/FHA-expressing B. pertussis strains developed serum and mucosal anti-FHA and anti-TbpB antibodies (in BALF) [41]. The serum anti-TbpB antibodies were of the IgG1, IgG2a and IgG2b classes [41]. Interestingly there was no significant difference in the antibody responses to FHA or TbpB in BALF or serum samples from mice immunised with PTX+ or PTX—strains [41]. The mouse is not a good animal model of N. meningitidis infection; however, mouse anti-TbpB antibodies were demonstrated to be bactericidal for N. meningitidis in vitro which correlates with protective immunity in humans [41]. The system was also shown to be efficient for delivery of a serologically different N. meningitidis TbpB [41].

It has been widely reported that induction of a specific immune response by vaccinating at one mucosal surface can result in immune responses to the same antigen at other, remote mucosal sites [42]. i/n immunisation with mucosal vaccines has been shown to stimulate specific immune responses in the respiratory tract and in the female genital tract [43]. Therefore it may be possible to use i/n immunisation to protect against sexually transmitted diseases. Mice immunised i/n with either wild-type B. pertussis or B. pertussis ptx—have been shown to produce significant levels of anti-FHA IgG and IgA in vaginal secretions [44].

A non-toxic region of tetanus toxin called fragment C (FrgC) has been used as a model heterologous antigen in a variety of live bacterial vectors [45–55]. FrgC comprises the C-terminal 50 kDa of tetanus toxin. There are probably a number of reasons why FrgC has been so widely studied as a heterologous antigen. FrgC, natural or recombinant, is a good protective immunogen when administered parenterally but is generally a poor mucosal immunogen [56]. FrgC can be expressed at high levels intracellularly in a number of heterologous organisms (both eukaryotic and prokaryotic). There is a very good murine model of immunity to tetanus that requires small numbers of animals, is of short duration (less than a week following challenge) and quantitative [57]. There are also important medical reasons for using FrgC. Tetanus is an important disease in humans and a number of animal species.

Despite the existence of effective vaccine against tetanus, over half a million people die from tetanus annually, most of these deaths occurring in the third world [58]. A tetanus vaccine which could be administered at a mucosal surface, circumventing the use of needles, and that could be stored without a cold chain would represent a considerable advance.

The current tetanus vaccine (tetanus toxoid) is usually combined with diphtheria vaccine (diphtheria toxoid) and whooping cough vaccine (whole cell or acellular) to produce DTP vaccine. Immunity to tetanus and diphtheria depends on a single antigen whereas effective immunity to B. pertussis requires multiple large, complex antigens, such as PTX, pertactin, FHA and fimbriae [11]. Therefore expressing non-toxic forms of tetanus toxin (such as FrgC) and diphtheria toxin (such as CRM197) in an attenuated B. pertussis strain is probably a more feasible and practical approach to developing a mucosal DTP vaccine than using a different bacterial vector strain such as an attenuated Salmonella to express tetanus, diphtheria and multiple B. pertussis antigens.

FrgC was expressed in B. pertussis fused to Fha44. A plasmid encoding the FrgC/FHA fusion was rapidly lost from B. pertussis carrier strains in vivo [59]. The hybrid gene was incorporated into the chromosome of B. pertussis to overcome the problem of gene instability. However, the level of expression of the FrgC/FHA protein was low and in addition the fusion molecule was unstable [59]. Even after two i/n immunisations with either PTX+ or PTX—strains of B. pertussis expressing FrgC/FHA, anti-FrgC antibodies could not be detected [59]. Mice were primed for an anti-FrgC response because they mounted a serum anti-FrgC antibody response when they were boosted i/n with purified FrgC [59].

3.5. B. bronchiseptica as a vector for heterologous antigens

Attenuated B. bronchiseptica have also been used to
deliver FrgC to the respiratory tract of mice. FrgC has been expressed in the *B. bronchiseptica* aro*A* strain from a broad host range vector under the control of two different promoters, both of these enterobacterial promoters that have been used to express FrgC in attenuated *Salmonella* spp. [36]. The first of these promoters, *Ptac*, constitutively expresses FrgC in *B. bronchiseptica* [36]. The second, *PfrtA*, is a stress response promoter that is environmentally regulated [57]. Both of these constructs are relatively stable in *B. bronchiseptica* in vivo, 3 days post i/n immunisation approximately 70% of bacteria isolated from mouse lungs still harbouring the plasmid (unpublished data). The highest levels of anti-FrgC serum antibody were detected in mice immunised i/n with *B. bronchiseptica* aro*A* strain expressing FrgC constitutively under control of *Ptac* (data not shown). Anti-FrgC serum antibody responses were detected in mice after immunisation with a single dose of this strain [36]. *B. bronchiseptica* aro*A* *Ptac*:FrgC (GVB120) was chosen for further study. Mice were immunised i/n with a 1× or 3×prime regime followed by a single boost [36]. Interestingly, although there was little difference in the serum anti-*B. bronchiseptica* antibody response in mice immunised by the two regimes, the 3×prime regime gave a much higher serum anti-FrgC antibody titre. Both IgG1 and IgG2a anti-FrgC antibodies were induced by immunisation; however, the IgG2a titres were much higher indicating that the immune response was biased towards a Th1-type response. Approximately 40% of animals immunised i/n with *B. bronchiseptica* aro*A* *Ptac*:FrgC by the 3×prime regime were protected from challenge with a lethal dose of tetanus toxin [36]. Survival after tetanus toxin challenge correlated with anti-FrgC serum titres of individual mice [36].

3.6. Other live bacterial strains for i/n delivery of foreign antigens

A variety of other bacterial species have been investigated as vectors for i/n delivery of heterologous antigens. These include non-pathogenic bacteria such as *Streptococcus* gordoni and *Staphylococcus carnosus*, GRAS (generally regarded as safe) bacteria such as *Lactococcus* spp., *Lactobacillus* spp. and attenuated strains of pathogens such as *Salmonella* spp. and BCG. For a recent review of the relative merits of these different bacteria as i/n antigen delivery systems see [5].

4. Future prospects

The use of *Bordetella* spp. as live vaccines and vectors for foreign antigens is in its infancy compared with other bacteria such as *Salmonella* spp. for which a wide range of attenuated strains and a variety of expression systems exist. A primary requirement is the construction of strains that are appropriately attenuated. *Bordetella aro*A mutants are very safe, but are probably too attenuated. The ideal vaccine/vector strain should be effective after a single dose. Inactivation of the *ptx* operon attenuates *B. pertussis* in mice as evidenced by the reduced local and systemic effects in mice infected with PTX− strains. However, the role of PTX in pertussis pathogenesis in humans is controversial [11]. So it is likely that additional mutations would have to be included in *B. pertussis* *ptx* mutants before they would be acceptable for clinical use.

As mentioned previously, *Bordetella* spp. possess all of the known bacterial protein secretion systems so it should be feasible to secrete all types of foreign antigens if required. For example the adenylate cyclase toxin (ACT) is secreted by a type I secretion system and ACT has been used to deliver cytotoxic T-cell epitopes into the cytoplasm of eukaryotic cells [60,61].

One thing that has been shown to be detrimental to the efficacy of many different live vector systems is pre-existing immunity to the carrier strain [62]. At least for *B. pertussis*, it is unlikely for the foreseeable future that a live vaccine will replace immunisation with acellular or killed whole cell vaccines. Therefore individuals who may receive a live *B. pertussis* vaccine are likely to have pre-existing immunity to *B. pertussis* (although this immunity wanes by age). In the case of *B. bronchiseptica* it is possible that animals will have immunity to *B. bronchiseptica* either through infection or through prior immunisation. Since *B. bronchiseptica* can persist for prolonged periods in the respiratory tract, particularly the nasal cavity, in the face of high titres of anti-*B. bronchiseptica* antibodies this may mean that the efficacy of live *B. bronchiseptica* vectors may not be adversely affected by pre-existing immunity [36]. It has been shown with *Salmonella* delivery systems that the negative effect that pre-existing immunity to the *Salmonella* strain has on the response to the foreign antigen can be overcome (at least partially) by using more efficient in vivo promoters to direct expression of the foreign antigen [62]. The effect of prior immunity to *Bordetella* spp. on the immune responses of the host to heterologous antigens will need to be investigated experimentally.

The recently completed *B. bronchiseptica*, *B. pertussis* and *B. parapertussis* genome projects (http://www.sanger.ac.uk/Projects/B_pertussis/, http://www.sanger.ac.uk/Projects/B_bronchiseptica/ and http://www.sanger.ac.uk/Projects/B_parapertussis/) and the information that will accrue from the utilisation of this information in functional genomics and comparative studies of these organisms should provide an invaluable resource for selecting genes to target for constructing the next generation of candidate live *Bordetella* vaccine strains. It will also allow us to optimise the expression of important antigens within these systems, increasing the overall efficacy of i/n immunisation with *Bordetella* spp. with the ultimate goal of producing a single-dose, multivalent respiratory vaccine.
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References


