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Bacillus subtilis as a tool for vaccine development: from antigen factories to delivery vectors

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ABSTRACT

Bacillus subtilis and some of its close relatives have a long history of industrial and biotechnological applications. Search for antigen expression systems based on recombinant *B. subtilis* strains sounds attractive both by the extensive genetic knowledge and the lack of an outer membrane, which simplify the secretion and purification of heterologous proteins. More recently, genetically modified *B. subtilis* spores have been described as indestructible delivery vehicles for vaccine antigens. Nonetheless both production and delivery of antigens by *B. subtilis* strains face some inherent obstacles, as unstable gene expression and reduced immunogenicity that, otherwise, can be overcome by already available gene technology approaches. In the present review we present the status of *B. subtilis*-based vaccine research, either as protein factories or delivery vectors, and discuss some alternatives for a better use of genetically modified strains.

Key words: vaccines, *Bacillus subtilis*, immunization, protein expression.

INTRODUCTION

Besides the well-known reputation in the industrial production of enzymes, such as proteases and amylases, some *Bacillus* species, with particular emphasis on *B. subtilis*, have been explored as a host for the expression of foreign proteins with pharmacological or immunological activities (Harwood 1992, Wong 1995). Such interest stems from a plethora of very solid reasons: (i) as gram-positive bacteria, *B. subtilis* strains do not have an outer membrane and, thus, all secreted proteins are released directly into the growth medium, which greatly simplifies and reduces the costs of downstream purification steps; (ii) the available knowledge on genetics and physiology of *B. subtilis* finds parallel only with *Escherichia coli* K12, making easier the development of controllable gene expression systems and adaptation to large-scale stream-line fermentation processes; (iii) *B. subtilis* strains have a well-established safety record and have deserved the GRAS (*generally regarded as safe*) status; (iv) production of spores, the most resistant life form found on earth, warrants easy preservation of strains even under harsh environmental conditions; and, finally, (v) the ability to grow in simple and non-expensive media at fast growth rates and a non-biased codon usage confer to this bacterial species a top candidate position for the expression of heterologous proteins, including those with potential use in vaccine development (Henner 1990, Wong 1995).

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Modern vaccine development approaches have employed genetically modified bacteria in three distinct ways. First live vaccines based on attenuated pathogens can be obtained after introduction of mutations, usually deletion of genes required for the ability to grow or inflict damage into the mammal host (Hormaeche et al. 1999). Second, bacterial strains can be converted into convenient and low cost cellular factories for production of bioactive molecules such as proteins, polysaccharides and nucleic acids. Such compounds can be purified by genetically modified strains and incorporated as antigens into subunit-based vaccine formulations (Vilar et al. 2003). Finally, some bacterial species can be genetically modified in order to become live carriers delivered either by parenteral or, preferentially, by mucosal routes, of passenger antigens derived from one or several pathogens, either as intact proteins or peptides genetically fused to host bacterial proteins (Medina and Guzman 2001, Curtiss et al. 1989).

So far attempts to use *B. subtilis* strains in vaccine development have focused mainly on the production of recombinant antigens, both as intracellularly expressed proteins or soluble proteins secreted into the extra-cellular medium. More recently, particular attention was given to the use of genetically modified *B. subtilis* spores expressing surface-exposed antigens genetically fused to spore coat proteins. In this review, both approaches will be considered and some positive and negative aspects will be discussed taking into account the possible impact of this technology on the field of vaccine development.

PRODUCTION OF RECOMBINANT ANTIGENS BY *B. subtilis* STRAINS

Modern subunit vaccines require production of highly purified antigens, which can be more easily achieved by genetically modified bacterial or yeast strains, as successfully illustrated by the recombinant hepatitis B vaccine (McAleer et al. 1984). The use of a recombinant microbial host avoids direct contact with the pathogen and usually allows reproducible and high recovery yields at much reduced costs. So far production of antigens for vaccine use have been mainly based on yeast or *E. coli* K12 strains as cell factories (Cereghino and Cregg 2000, Cornelis 2000). Nonetheless, some *Bacillus* species, particularly *B. subtilis* strains, have been already employed in the production of heterologous antigens due to both the availability of several well-established expression systems and the ability to secrete recombinant proteins into the culture medium. In spite of these attractive features only a small number of research groups have consistently worked with recombinant *B. subtilis* strains as hosts for expression of antigens with potential vaccine application in humans (Table I).

The protective antigen (PA) of *B. anthracis* is a major protective antigen and is included in the presently available acellular anthrax vaccine (Belton and Strange 1954, Puziss et al. 1963). As a common receptor-binding domain of two *B. anthracis* toxins, lethal factor and edema factor, PA represents the main target of specific neutralizing antibodies that confer the protective status to the presently available vaccines both for human and veterinary use (Baillie 2001, Friedlander et al. 1999, 2002). As a protein produced by a highly pathogenic *Bacillus* species, the expression and purification of the PA antigen by recombinant *B. subtilis* strains represented a safer choice for the production of anti-anthrax acellular vaccines. The PA-encoding sequence was cloned in expression plasmids and successfully expressed and secreted by recombinant strains (Baillie et al. 1994, Ivins and Welkos 1986, McBride et al. 1998). Recombinant PA purified from culture supernatants of *B. subtilis* cultures proved to be fully immunogenic and conferred protection both to mice and Guinea pigs challenged with lethal doses of *B. anthracis* spores (Ivins and Welkos 1986, McBride et al. 1998). An expression system based on an inducible *B. subtilis* lysogenic phage has also been employed to express a secreted and immunogenic form of the *B. anthracis* PA (Baillie et al. 1994).

Plasmid-based *B. subtilis* expression systems have also been successfully applied in the production of pertussis toxin (PT) subunits and the

TABLE I

Antigens expressed by recombinant *B. subtilis* strains aiming the production of vaccines.

Expressed antigen	Target pathogen	Expression system	Immune response or protection	Reference
Production of recombinant antigens for subunit vaccines				
PA	*Bacillus anthracis*	Plasmid-encoded secreted protein	Protective serum antibody response	[Ivins and Welkos 1986, McBride et al. 1998, Baillie et al. 1994]
		Secreted protein encoded by inducible lysogenic phage	Protective serum antibody response	[Baillie et al. 1998]
Pneumolysin	*Streptococcus pneumonia*	Plasmid-encoded secreted and cytoplasmic proteins	Antigenicity in immunoassays	[Taira et al. 1989]
P1	*Neisseria meningitidis*	Plasmid-encoded inclusion bodies	Bactericidal serum antibody response	[Nurminen et al. 1992, Idänpään-Heikkila et al. 1995, 1996]
OmpP2	*Haemophilus influenzae*	Plasmid-encoded cytoplasmic protein	Non-protective antibody responses	[Srikumar et al. 1993]
PT subunits	*Bordetella pertussis*	Plasmid-encoded secreted and cytoplasmic proteins	Serum antibody responses	[Saris et al. 1990, Himanen et al. 1990]
Omps and Hsp60	*Chlamydia pneumonia*	Plasmid-encoded cytoplasmic proteins	Antibody and lymphoproliferation responses	[Airaksinen et al. 2003]
Spare-based delivery systems				
TT-C	*Clostridium tetani*	Chromosomal-encoded C-terminal fusion with spore coat CotB/C proteins	Protective serum antibody responses	[Isticato et al. 2001, Duc et al. 2003a, Mauriello et al. 2004]
LTB	*Escherichia coli* (ETEC)	Chromosomal-encoded C-terminal fusion with spore coat CotC protein	Induction of serum and fecal antibody responses	[Mauriello et al. 2004]

Neisseria meningitides class I outer membrane protein (P1), either as soluble secreted or intracellular insoluble proteins (Idänpään-Heikkila et al. 1995, 1996, Himanen et al. 1990, Nurminen et al. 1992, Saris et al. 1990). In both cases protective antibody responses were achieved in animals immunized with the purified proteins but additional steps had to be included in order to enhance or activate the immunogenicity of the encoded antigens. In the case of the P1 protein, incorporation into liposomes or detergent micelles were required for the stabilization of the rather hydrophobic outer membrane protein, while a refolding step was required after denaturing of pertussis toxin subunits from inclusion bodies (Idänpään-Heikkila et al. 1995, 1996, Himanen et al. 1990, Nurminen et al. 1992). In both cases the reduced immunogenicity of the antigens expressed by the *B. subtilis* strains was attributed to the lack of conformational epitopes, lost during expression or purification steps, required for induction of protective antibody responses.

Three *Chlamydia pneumoniae* proteins, two outer membrane proteins and one cytoplasmic heat-shock inducible protein, were cloned in expression plasmid vectors and produced in *B. subtilis* strains either as insoluble (membrane proteins) or soluble

cytoplasmic proteins with histidine tags (Airaksinen et al. 2003). Purified proteins elicited specific serum antibodies, after parenteral administration to mice, which reacted with the native proteins expressed by *C. pneumonia* strains and induced cell proliferation after exposure of splenocytes to elementary bodies (Airaksinen et al. 2003). Other reports have also described the use of recombinant *B. subtilis* strains for the production of pneumolysin, a pneumococcal toxin, and an outer membrane protein of *Haemophilus influenzae*, either as secreted or cytoplasmic proteins, encoded by multi-copy plasmid-based expression systems (Srikumar et al. 1993, Taira et al. 1989). Both proteins were suitable for immunological assays but, in the case of the *H. influenzae* porin, the altered conformation of the recombinant protein preclude the generation of neutralizing antibodies (Taira et al. 1989).

The rather restricted list of proteins expressed by *B. subtilis* strains aiming the development of subunit vaccines contrasts with the potential advantages of the *Bacillus* model as a host for protein expression. At least in part, the reduced interest on the use of *B. subtilis* as a host for protein expression can be ascribed to commercially available expression systems based in *E. coli* K12 of yeast systems (Cereghino and Cregg 2000, Studier et al. 1990). Moreover, the reduced recovery yields obtained with some expression systems have been frequently cited as an argument for the low interest for *B. subtilis* as a protein factory (Saris et al. 1990, Himanen et al. 1990, Taira et al. 1989). Reduced expression levels of heterologous proteins by recombinant *B. subtilis* strains may be ascribed to three main factors. First, the unstable gene expression, associated either with the loss of the recombinant plasmid (segregational instability) or rearrangement of the cloned gene (structural instability), may reduce gene expression by loss of the genetic information (Bron et al. 1988, Ehrlich et al. 1986). Most *B. subtilis* strains produce large amounts of extracellular proteases, which can quickly degrade most the foreign proteins produced and secreted by the recombinant strains (Wang et al. 1988). Finally, the limited number of promoters available for construction of plasmid expression vectors restricted the development of strains with a better performance as cell factories for recombinant proteins. In all three situations, technical solutions for each problem have already been obtained and are available for use in heterologous protein expression.

Integration of the heterologous gene into specific sites of the *B. subtilis* chromosome has been frequently employed to enhance gene expression stability. The presence of sequences sharing sequence homologies with target chromosomal genes and selective antibiotic markers allows the integration of recombinant genes under the control of different promoters after allelic exchange by homologous recombination (Härtl et al. 2001, Mogk et al. 1996). Stable gene expression in *B. subtilis* can also be achieved with plasmids replicating via double-strand intermediates, similarly to plasmids derived from gram-negative bacteria (Jannière et al. 1990). In contrast to chromosome integration methods, enhanced structural and segregational gene expression could be achieved with such vectors without reduction of gene copy number (Jannière et al. 1990). The identification of secreted and cell-bound proteases of *B. subtilis* permitted the generation of strains defective in 6, 7 or 8 different proteases, which greatly improved the recovery of recombinant proteins from the growth medium of genetically modified strains (Wu et al. 1991, 2002). The complete sequencing of the *B. subtilis* genome and the availability of promoter trap systems greatly simplified the task of identification and evaluation of promoters with potential applications for heterologous gene expression (Gat et al. 2003). Based on the evidences cited above, development of efficient antigen expression systems is clearly feasible with the available knowledge of *B. subtilis*.

B. subtilis SPORES AS LIVE CARRIERS OF ANTIGENS

Both gram-negative and -positive bacteria have been extensively used as live carriers of vaccine antigens. Such strains can be delivered via the

oral route avoiding the use of needles and eliminating the risk of iatrogenic transmission of infectious diseases. In addition, there is no need to purify the expressed antigens, which are produced by the biosynthetic apparatus of the carrier strain. Given by the oral route, such live vaccines can induce both mucosal and systemic immune responses to endogenous and heterologous antigens. They can have a bi- or multivalent character according to the number of expressed antigens and the genetic background of the bacterial host. In contrast to the gram-negative hosts, which are usually represented by attenuated strains of enteric pathogens such as *Salmonella* and *Shigella* species (Curtiss 2002, Curtiss et al. 1989, Medina and Guzman 2001), most gram-positive species used as live carrier of vaccine antigens are harmless posing no potential risk to humans or animals (Pozzi and Wells 1997). Indeed most of the gram-positive bacterial species employed as oral vaccine antigen carriers belong to our natural microbiota and/or take part of our daily meals, as lactic bacteria including *Streptococcus gordoni*, *Lactococcus lactis*, and several *Lactobacillus* species (Pozzi and Wells 1997, Seegers 2002). As an additional appealing feature, some gram-positive bacterial species used as live carrier of vaccine antigens also display a probiotic action and, therefore, may play a dual role on the treatment and prevention of enteric infections (Holzapfel and Schillinger 2002).

The use of genetically modified *B. subtilis* strains as live carriers of vaccine antigens was recently reported and, so far, has relied on the unique feature of a few gram-positive bacteria, the ability to form endospores. Bacterial endospores represent the most resistant life form found on our planet and potentially would have an endless shelf-live (Nicholson et al. 2000). Moreover, *B. subtilis* spores have also a record of therapeutic application as probiotics both for humans and animals (Mazza 1994). The initial reports that recombinant *B. subtilis* spores could carry surface-exposed antigens and induce both systemic and secreted antigen-specific antibody responses after oral delivery to mice were received as new and promising alternative for development of oral vaccines (reviewed by Duc and Cutting 2003, Oggioni et al. 2003, Ricca and Cutting 2003).

Two spore coat proteins, CotB and CotC, have been used to display tetanus toxin fragment C (TTC) of *Clostridium tetani* and the B subunit of the heat labile toxin (LTB) produced by some enterotoxigenic *E. coli* strains (ETEC) as fused proteins expressed on the surface of *B. subtilis* spores (Isticato et al. 2001, Duc et al. 2003a, Mauriello et al. 2004) (Table I). Both antigens were expressed as C-terminal fusions with the Cot proteins, which are by themselves anchored at the outer layer of the spore coat at a number of approximately 1,000 molecules/spore (Isticato et al. 2001, Duc et al. 2003a). In contrast to the attempts to employ *B. subtilis* strains as expression systems of heterologous proteins for subunit vaccines, the genes encoding hybrid spore coat proteins were integrated into specific sites at the bacterial chromosome, which conferred stability to the expression of the recombinant genes (Duc et al. 2003a, Isticato et al. 2001, Mauriello et al. 2004). Mice orally treated with three consecutive daily doses of recombinant spores, given three times at intervals of two weeks, developed statistically significant secreted fecal (IgA) and serum (IgG) antibody levels which, at least in the case of TTC, could confer protection to mice challenged with lethal doses of the toxin (Duc et al. 2003a, Mauriello et al. 2004).

In spite of the clear interest in the development of a new mucosal vaccine delivery method that could combine both prophylatic and therapeutic effects in an indestructible carrier particle, there are some points which clearly demand further improvements before *B. subtilis* spores could be efficiently employed as live carriers of vaccine antigens. At present, the main concern is the low immunogenicity of *B. subtilis* spores in mammal hosts. In contrast to *B. anthracis*, *B. subtilis* spores do not efficiently activate strong local or systemic antibody responses, against itself and carrier proteins, particularly when delivered via the oral route (Duc et al.

2003a, Mauriello et al. 2004). Such features could be attributed to the ubiquitous presence of spores in the environment and its chemical composition that is devoid of compounds known to induce inflammatory responses in mammal hosts. Thus, oral immunization regimens with recombinant *B. subtilis* spores require high antigen loads (above 10^{10} spores/dose) and repeated immunizations (Duc et al. 2003a, Mauriello et al. 2004). Indeed some experimental evidences suggested that most of the local and systemic immunogenicity of recombinant *B. subtilis* spores is triggered during the intracellular germination and transient persistence of the nascent vegetative cell in the phagosome of phagocytic cells, as macrophages (Duc et al. 2003b, 2004).

OTHER *Bacillus* SPECIES EMPLOYED IN VACCINE DEVELOPMENT

B. brevis strains have been successfully used as a host for the production of recombinant proteins with biotechnological or pharmaceutical interest such as cytokines, hormones and single chain antibodies, based on multicopy plasmid expression systems able to promote secretion of encoded proteins into the growth media (Kozuka et al. 1996, Udaka et al. 1989, Udaka and Yamagata 1993, Shiroya et al. 2001, Takimura et al. 1997). Of particular interest for the vaccine field was the production of recombinant toxin subunits of *B. pertussis* and the LTB subunit of LT produced by ETEC strains, to be used either as antigens for subunit vaccines or as a mucosal adjuvant in the case of LTB, by recombinant *B. brevis* strains (Byun et al. 2001, Kozuka et al. 2000). Nonetheless, in contrast to the *B. subtilis* expression systems, recombinant proteins expressed and secreted in *B. brevis* strains usually require rather long cultivation periods (from 5 to 8 days) in order that maximal expression levels could be achieved (Byun et al. 2001, Kozuka et al. 2000).

A considerable increase in the interest to develop new and more efficient anthrax vaccines has been observed as a direct response to the growing attention to the terrorist threat (Baillie 2001, Friedlander et al. 1999, 2002). *B. anthracis* vaccines based on the spores of an attenuated strain or as a cell-free vaccine are already available both for humans and animals since many years (Barnard and Friedlander 1999, Cohen et al. 2000). Nonetheless, some research groups have evaluated the behavior of attenuated *B. anthracis* strains as live carriers of antigens derived from other gram-positive bacterial antigens (Mesnage et al. 1999, Sirard et al. 1997a, b). The Ib component of iota toxin from *Clostridium perfringens*, the listeriolysin from *Listeria monocytogenes* and the fragment C of tetanus toxin were successfully expressed by vegetative cells of a *B. anthracis* vaccine and parenteral administration of 10^8 spores at a single or two dose-based immunization regimens elicited high and protective antigen-specific systemic antibody responses (Mesnage et al. 1999, Sirard et al. 1997a, b). In spite of the excellent immunogenicity in laboratory animal models, clinical use of *B. anthracis* bivalent vaccine strains seems to be remote but the vaccine system have a clear interest for veterinary use (Brossier et al. 1999).

IMPROVING THE USE OF *B. subtilis* IN VACCINE DEVELOPMENT

The available evidences indicate that genetically modified *B. subtilis* strains, as well as other *Bacillus* species, can represent useful tools for the vaccine research field either as cell factories or live carriers of heterologous antigens. However, both applications demand technical improvements to enhance gene expression and/or immunogenicity of the produced antigens (Table II). At present, most of the technical problems faced by those who had employed *B. subtilis* strains for the production of vaccine antigens have already been surpassed, as discussed above. Expression systems located either on stable plasmids (Titok et al. 2003) or allowing ectopic integration at different sites of the chromosome (Guérout-Fleury et al. 1996, Härtl et al. 2001, Middleton and Hofmeister 2004, Shimotsu and Henner 1986) are available for *B. subtilis* and, some of its close relatives such as *B. brevis*. Several antigens have been successfully expressed and secreted into the growth medium in their biological active form amenable

TABLE II

Improving the use of *B. subtilis* as a tool for vaccine development.

As a cell factory of antigens for subunit vaccines
• Gene expression stabilization by chromosome integration of the recombinant genes or use of structurally stable multi-copy plasmids
• Identification of new promoters or/and optimization of existing promoters allowing high level expression and optimal secretion of encoded antigens
• Protease-deficient *B. subtilis* strains as hosts for antigen production
As live carriers of orally delivered antigens
• Strains able to germinate earlier and persist longer in phagocytic cells
• Strains with enhanced resistance to the gastrointestinal environment
• Development of expression systems able to drive gene expression under stress conditions
• Co-administration of mucosal adjuvants

either for biotechnological or biochemical applications (Simonen and Palva 1993, Udaka et al. 1989, Udaka and Yamagata 1993, Wang et al. 1988, Wong 1995, Wu et al. 2002, Wu and Wong 1999). Strong promoters with tightly regulated activity, as those induced by xylose or IPTG, are available for *B. subtilis* and some of them have already been tested for expression of heterologous or endogenous proteins either as intracellular or secreted proteins (Conrad et al. 1996, Kim et al. 1996). Similarly, *B. subtilis* strains unable to secrete several proteases have been shown to reduce degradation of secreted heterologous proteins to trace levels, as compared to the previously used laboratory strains, and can be used as more appropriate hosts for the production of vaccine antigens (Wu et al. 1991, 2002).

Although promising as a vaccine tool, application of *B. subtilis* as an antigen delivery vehicle, either as spores or vegetative cells, will demand a better understanding of the fate of cells and spores after oral ingestion or parenteral administration to mammal hosts. Definition of specific events relevant in the triggering of humoral and cellular immune responses can contribute to the development of more rational and efficient vaccine strategies with enhanced immunogenicity. Recent evidences indicate that germination of *B. subtilis* spores located into phagosomes is a key step on the activation of immune responses since it permit antigen presenting cells to sample, process and display peptides to lymphocytes (Hoa et al. 2001). Similar events seem to occur with the spores of the more immunogenic *B. anthracis* vaccine strains, which germinate and transiently multiply inside macrophages (Cohen et al. 2000). Thus, development of *B. subtilis* strains with enhanced ability to elicit immune responses to passenger antigens shall explore mimic some *B. anthracis* features as early and more efficient intracellular spore germination, ability to transiently survive and multiply inside phagocytic cells or in vivo express antigens more efficiently after phagocytosis.

Expression of listeriolysin has allowed phagosome escape and transient intracellular multiplication of recombinant *B. subtilis* (Bielecki et al. 1990, Hoa et al. 2001). Mutations allowing early germination of *B. subtilis* spores are known but the role during the transit into the mammal host is unknown (Setlow 1994). Finally, hundreds of genes activated during exposure to stressful conditions, as low pH or anaerobiosis, are controlled by alternative sigma factor and their promoters could be use to drive the *in vivo* expression of antigens (Schumann 2003, Wiegert et al. 2001). Similarly strains able to constitutively express σ^{B}-dependent stress regulon

may better resist the transit through the gastrointestinal tract as vegetative cells (Völker et al. 1999). Finally, mucosal and systemic immunogenicity of both spores and vegetative cells may be increased by co-administration of mucosal adjuvants as CT or LT (Bowman and Clements 2001, Guillobel et al. 2000). Such experimental alternatives can be easily tested and might contribute to the more efficient use of *B. subtilis* strains as vaccine carries.

THE FUTURE OF *B. subtilis* AS A TOOL FOR VACCINE DEVELOPMENT

Although some of the most important diseases that scourged mankind during centuries have been eradicated or kept under control by the use of vaccines, several diseases causing millions of casualties are still among us without an efficient vaccine alternative. The search of new vaccines has experienced an extraordinary increase during the last two decades and, at present, hundreds of distinct vaccine formulations are under laboratory testing or clinical evaluation. With this notion in mind, the use of *B. subtilis* strains, either as a host for production of purified antigens or as orally-delivered carrier of vaccine antigens, may find a more broad interest especially for diseases affecting the gastrointestinal tract, which can also obtain benefits from the probiotic nature of spores. The foundations of *B. subtilis*-based vaccines have been settled and the instruments to achieve a more efficient performance of the system are known. It is now a question of persistence and dedication of those interested in the field to obtain new *B. subtilis* strains that could fulfill more efficiently the task.

RESUMO

Bacillus subtilis e alguns de seus parentes mais próximos possuem uma longa história de aplicações industriais e biotecnológicas. A busca de sistemas de expressão de antígenos baseados em linhagens recombinants de *B. subtilis* mostra-se atrativa em função do conhecimento genético disponível e ausência de uma membrana externa, o que simplifica a secreção e a purificação de proteínas heterólogas. Mais recentemente, esporos geneticamente modificados de *B. subtilis* foram descritos com veículos indestrutíveis para o transporte de antígenos vacinais. Todavia a produção e o transporte de antígenos por linhagens de *B. subtilis* encontra obstáculos, como a expressão gênica instável e imunogenicidade reduzida, que podem ser superados com o auxílio de tecnologias genéticas atualmente disponíveis. Apresentamos nesta revisão o estado atual da pesquisa em vacinas baseadas em *B. subtilis*, empregado tanto como fábrica de proteínas ou veículos, e discute algumas alternativas para o uso mais adequado de linhagens geneticamente modificadas.

Palavras-chave: vacinas, *Bacillus subtilis*, imunização, expressão de proteínas.

REFERENCES

Airaksinen U, Penttilä T, Wahlström E, Vuola JM, Puolakkainen M and Sarvas M. 2003. Production of *Chlamydia pneumonia* proteins in *Bacillus subtilis* and their use in characterization of immune responses in the experimental infection model. Clin Diag Lab Immunol 16: 367–375.

Baillie L. 2001. The development of new vaccines against *Bacillus anthracis*. J Appl Microbiol 91: 609–613.

Baillie LW, Johnson M and Manchee RJ. 1994. Evaluation of *Bacillus subtilis* strain IS53 for the production of *Bacillus antracis* protective antigen. Let Appl Microbiol 19: 225–227.

Baillie LWJ, Moore P and McBride BW. 1998. A heat-inducible *Bacillus subtilis* bacteriophage F105 expression system for the production of the protective antigen of *Bacillus anthracis*. FEMS Microbiol Let 163: 43–47.

Barnard JP and Friedlander AM. 1999. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. Infect Immun 67: 562–567.

Belton FC and Strange RE. 1954. Studies on a protective antigen produced *in vivo* from *Bacillus anthracis* medium and methods of production. Brit J Exp Pathol 35: 114–152.

Bielecki J, Youngman P, Connelly P and Portnoy DA. 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. Nature 345: 175–176.

Bowman CC and Clements JD. 2001. Differential biological and adjuvant activities of cholera toxin and

Escherichia coli heat-labile enterotoxin hybrids. Infect Immun 69: 1528–1535.

Bron S, Luxen E and Swart P. 1988. Instability of recombinant pUB110 plasmids in *Bacillus subtilis*: plasmid-encoded stability function and effects of DNA inserts. Plasmid 19: 231–241.

Brossier F, Mock M and Sirard JC. 1999. Antigen delivery by attenuated *Bacillus anthracis*: new prospects in veterinary vaccines. J Appl Microbiol 87: 298–302.

Byun Y, Ohmura M, Fujihashi K, Yamanoo S, McGhee JR, Udaka S, Kiyono H, Takeda Y, Kohsaka T and Yuki Y. 2001. Nasal immunization with *E. coli* verotoxin 1 (VT1)-B subunit and a nontoxic mutant of cholera toxin elicits serum and neutralizing antibodies. Vaccine 19: 2061–2070.

Cereghino JL and Cregg JM. 2000. Heterologous protein expression in methylotrophic yeat *Phichia pastoris*. FEMS Microbiol Rev 24: 45–66.

Cohen S, Mendelson I, Altboum Z, Kobiler D, Elhanany E, Bino T, Leitner M, Inbar I, Rosenberg H, Gozes Y, Barak R, Fisher M, Kronman C, Velan B and Shafferman A. 2000. Attenuated nontoxinogenic and nonencapsulated recombinant *Bacillus anthracis* spore vaccines protect against anthrax. Infect Immun 68: 4549–4558.

Conrad B, Savchenko RS, Breves R and Hofemeister J. 1996. A T7 promoter-specific, inducible protein expression system for *Bacillus subtilis*. Mol Gen Genet 250: 230–236.

Cornelis P. 2000. Expressing genes in different *Escherichia coli* compartments. Curr Op Biotech 11: 450–454.

Curtiss R III. 2002. Bacterial infectious disease control by vaccine development. J Clin Invest 110: 1061–1066.

Curtiss R III, Kelly SM, Gulig PA and Nakayama K. 1989. Selective delivery of antigens by recombinant bacterial. Curr Top Microbiol Immunol 146: 35–49.

Duc LH and Cutting SM. 2003. Bacterial spores as heat stable vaccine vehicles. Expert Opin Biol Ther 3: 1263–1270.

Duc LH, Hong HA, Fairweather N, Ricca E and Cutting SM. 2003a. Bacterial spores as vaccine vehicles. Infect Immun 71: 2810–2818.

Duc LH, Hong HA and Cutting SM. 2003b. Germination of the spore in the gastrointestinal tract provides a novel route for heterologous antigen delivery. Vaccine 21: 4215–4224.

Duc LH, Hong HA, Uyen NQ and Cutting SM. 2004. Intracellular fate and immunogenicity of *B. subtilis* spores. Vaccine 22: 1873–1885.

Ehrlich SD, Noirot P, Petit MA, Jannière L, Michel B and Riele HT. 1986. Structural instability of *Bacillus subtilis* plasmids. In: Setlow JK and Hollaender A. (Ed.), Genetic Engineering, vol. 8, New York, Plenum Press, p. 71–83.

Friedlander AM, Pittman PR and Parker GW. 1999. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. JAMA 282: 2104–2106.

Friedlander AM, Welkos SL and Ivins BE. 2002. Anthrax vaccines. Curr Top Microbiol Immunol 271: 33–60.

Gat O, Inbar I, Aloni-Grinstein R, Zahavy E, Kronman C, Mendelson I, Cohen S, Velan B and Shafferman A. 2003. Use of a promoter trap system in *Bacillus anthracis* and *Bacillus subtilis* for the development of recombinant protective antigen-based vaccines. Infect Immun 71: 801–813.

Guérout-Fleury AM, Frandsen N and Stragier P. 1996. Plasmids for ectopic integration in *Bacillus subtilis*. Gene 180: 57–61.

Guillobel HC, Carinhanha JI, Cárdenas L, Clements JD, de Almeida DF and Ferreira LCS. 2000. Adjuvant activity of a nontoxic mutant of *Escherichia coli* heat-labile enterotoxin on systemic and mucosal immune responses elicited against a heterologous antigen carried by a live *Salmonella enterica* Serovar Typhimurium vaccine strain. Infect Immun 68: 4349–4353.

Härtl B, Wehrl W, Wiegert T, Homuth G and Schumann W. 2001. Development of a new integration site within the *Bacillus subtilis* chromosome and construction of compatible expression cassettes. J Bacteriol 183: 2696–2699.

Harwood CR. 1992. *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. Trends Biotech 10: 247–256.

Henner DJ. 1990. Expression of heterologous genes in *Bacillus subtilis*. Meth Enzymol 185: 199–201.

HIMANEN JP, TAIRA S, SARVAS M, SARIS P AND RUNEBERG-NYMAN K. 1990. Expression of pertussis toxin subunit S4 as an intracytoplasmic protein in *Bacillus subtilis*. Vaccine 8: 600–604.

HOA TT, DUC LH, ISTICATO R, BACCIAGALUPI L, RICCA E, VAN PH AND CUTTING SM. 2001. Fate and dissemination of *Bacillus subtilis* spores in a murine model. Appl Environ Microbiol 67: 3819–3823.

HOLZAPFEL WH AND SCHILLINGER U. 2002. Introduction to pre-and probiotics. Food Res Intern 35: 109–116.

HORMAECHE CE, JOYSEY HS, DE SILVA L, IZHAR M AND STOCKER BAD. 1999. Immunity induced by live attenuated *Salmonella* vaccines. Res Microbiol 141: 757–764.

IDÄNPÄÄN-HEIKKILA I, MUTTILAINEN S, WAHLSTRÖN E, SAARINEN L, LEINONEN M, SARVAS M AND MÄKELÄ PH. 1995. The antibody response to a prototype liposome vaccine containing *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis*. Vaccine 13: 1501–1508.

IDÄNPÄÄN-HEIKKILA I, WAHLSTRÖN E, MUTTILAINEN S, NURMINEN M, KÄYTHY H, SARVAS M AND MÄKELÄ PH. 1996. Immunization with meningococcal class 1 outer membrane protein produced by *Bacillus subtilis* and reconstituted in the presence of Zwittergent or Triton X-100. Vaccine 14: 886–891.

ISTICATO R, CANGIANO G, TRAN HT, CIABATTINI A, MEDAGLINI D, OGGIONI MR, DE FELICE M, POZZI G AND RICCA E. 2001. Surface display of recombinant proteins on *Bacillus subtilis* spores. J Bacteriol 183: 6294–6301.

IVINS BE AND WELKOS SL. 1986. Cloning and expression of *Bacillus anthracis* protective antigen gene in *Bacillus subtilis*. Infect Immun 54: 537–542.

JANNIÈRE L, BRUAND C AND ERLICH SD. 1990. Structurally stable *Bacillus subtilis* cloning vectors. Gene 87: 53–61.

KIM L, MOGK A AND SCHUMANN W. 1996. A xylose-inducible *B. subtilis* integration vector and its application. Gene 181: 71–76.

KOZUKA S, YASUDA Y AND TOCHIKUBO K. 1996. Expression and secretion of the S2 subunit of pertussis toxin in *Bacillus brevis*. Vaccine 14: 1707–1711.

KOZUKA S, YASUDA Y, ISAKA M, MASAKI N, TANIGUCHI T, MATANO K, MORIYAMA A, OHKUMA K, GOTO N, UDAKA S AND TOCHIKUBO K. 2000. Efficient extracellular production of recombinant *Escherichia coli* heat-labile enterotoxin B subunit by using the expression/secretion system of *Bacillus brevis* and its mucosal immunoadjuvanticity. Vaccine 18: 1730–1737.

MAURIELLO EMF, DUC LH, ISTICATO R, CANGIANO G, HONG HA, DE FELICE M, RICCA E AND CUTTING SM. 2004. Display of heterologous antigens on the *Bacillus subtilis* spore coat using CotC as a fusion partner. Vaccine 22: 1177–1187.

MAZZA P. 1994. The use of *Bacillus subtilis* as an antidiarrhoeal microorganism. Bol Chim Farm 133: 3–18.

MCALEER WJ, BUYNAK EB, MAIGETTER RZ, WAMPLER DE, MILLER WJ AND HILLEMAN MR. 1984. Human hepatitis B vaccine from recombinant yeast. Nature 307: 178–180.

MCBRIDE BW, MOGG A, TELFER JL, LEVER MS, MILLER J, TURNBULL PCB AND BAILLIE L. 1998. Protective efficacy of a recombinant protective antigen against *Bacillus anthracis* challenge and assessment of immunological markers. Vaccine 16: 810–817.

MEDINA E AND GUZMAN CA. 2001. Use of live bacterial vaccine vectors for antigen delivery: potential and limitations. Vaccine 19: 1573–1580.

MESNAGE S, LEVY MW, HAUSTANT M, MOCK M AND FOUET A. 1999. Cell surface-exposed tetanus toxin fragment C produced by recombinant *Bacillus anthracis* protects against tetanus toxin. Infect Immun 67: 4847–4850.

MIDDLETON R AND HOFMEISTER A. 2004. New shuttle vectors for ectopic insertion of genes into *Bacillus subtilis*. Plasmid 51: 238–245.

MOGK A, HAYWARD R AND SCHUMANN W. 1996. Integrative vectors for constructing single-copy transcriptional fusions between *Bacillus subtilis* promoters and various reporter genes encoding heat-stable enzymes. Gene 182: 33–36.

NICHOLSON WJ, MUNAKATA N, HORNECK G, MELOSH HJ AND SETLOW P. 2000. Resistance of *Bacillus* endospores to extreme terrestial and extraterrestrial environments. Microbiol Mol Biol Rev 64: 548–572.

NURMINEN M, BUTCHER S, IDÄNPÄÄN-HEIKKILA I, WAHLSTROM E, MUTTILAINEN S, RUNNERGNYMAN K, SARVAS M AND MAKELA PH. 1992. The class

1 outer membrane protein of *Neisseria meningitidis* produced by *Bacillus subtilis* can give rise to protective immunity. Mol Microbiol 6: 2499–2506.

Oggioni MR, Ciabattini A, Cuppone AM and Pozzi G. 2003. *Bacillus* spores for vaccine delivery. Vaccine 31: S2/96-S2/101.

Pozzi G and Wells JM. 1997. Gram-positive bacteria. Vaccine Vehicles for mucosal immunization. Springer-Verlag, Berlin, 180 p.

Puziss M, Manning LC, Lynch LW, Barclay E, Abelow I and Wright GG. 1963. Large-scale production of protective antigen of *Bacillus anthracis* anaerobic cultures. Appl Microbiol 11: 330–334.

Ricca E and Cutting SM. 2003. Emerging applications of bacterial spores in nanobiotechnology. J Nanotechnol 1: 6–15.

Saris P, Taira S, Airaksinen U, Palva A, Sarvas M and Palva I. 1990. Production and secretion of pertussis toxin subunits in *Bacillus subtilis*. FEMS Microbiol Let 68: 143–148.

Schumann W. 2003. The *Bacillus subtilis* heat shock stimulon. Cell Stress Chap 8: 207–217.

Seegers JFML. 2002. Lactobacilli as live vaccine delivery vectors: progress and prospects. Trends Biotechnol 20: 508–515.

Setlow P. 1994. Mechanisms which contribute to the long-term survival of spores of *Bacillus* species. J Appl Bacteriol 76: S49–S60.

Shimotsu H and Henner DJ. 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene 43: 85–94.

Shiroya T, Shibata Y, Hayakawa M, Shinozaki N, Fukushima K and Udaka S. 2001. Construction of a chimeric shuttle plasmid via a heterodimer system: secretion of an scFv protein from *Bacillus brevis* cells capable of inhibiting hemagglutination. Biosc Biotechnol Biochem 65: 389–395.

Simonen M and Palva I. 1993. Protein secretion in *Bacillus* species. Microbiol Rev 57: 109–137.

Sirard JC, Weber M, Duflot E, Popoff MR and Mock M. 1997a. A recombinant *Bacillus anthracis* strain producing *Clostridium perfringens* Ib component induces protection against iota toxins. Infect Immun 65: 2029–2033.

Sirard JC, Fayolle C, de Chastellier C, Mock M, Leclerc C and Berche P. 1997b. Intracytoplasmic delivery of listeriolysin O by a vaccinal strain of *Bacillus anthracis* induces CD8-mediated protection against *Listeria monocytogenes*. J Immunol 159: 4435–4443.

Srikumar R, Dahan D, Gras MF, Saarinen L, Kayhty H, Sarvas M, Vogel L and Coulton JW. 1993. Immunological properties of recombinant porin of *Haemophilus influenzae* type b expressed in *Bacillus subtilis*. Infect Immun 61: 3334–3341.

Studier FW, Rosenberg AH, Dunn JJ and Dubendorff JW. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth Enzymol 185: 60–89.

Taira S, Jalone E, Paton JC, Sarvas M and Runeberg-Nyman K. 1989. Production of pneumolysin, a pneumococcal toxin, in *Bacillus subtilis*. Gene 7: 211–218.

Takimura Y, Kato M, Ohta T, Yamagata H and Udaka S. 1997. Secretion of human interleukin-2 in biologically active form by *Bacillus brevis* directly into culture medium. Biosc Biotechnol Biochem 61: 1858–1861.

Titok MA, Chapuis J, Selezneva YV, Lagodich AV, Prokulevich VA, Ehrlich SD and Jannière L. 2003. *Bacillus subtilis* soil isolates: plasmid replicon analysis and construction of a new theta-replicating vector. Plasmid 49: 53–62.

Udaka S and Yamagata H. 1993. High-level secretion of heterologous proteins by *Bacillus brevis*. Meth Enzymol 217: 23–33.

Udaka S, Tsukagoshi N and Yamagata H. 1989. *Bacillus brevis*, a host bacterium for efficient extracellular production of useful proteins. Biotechnol Genet Engin Rev 7: 113–146.

Vilar MM, Barrientos F, Almeida M, Thaumaturgo N, Simpson A, Garrat R and Tendler M. 2003. An experimental bivalent peptide vaccine against schistosomiasis and fascioliasis. Vaccine 22: 137–144.

Völker U, Maul B and Hecker M. 1999. Expression of the σ^B-dependent general stress regulon confers multiple stress resistance in *Bacillus subtilis*. J Bacteriol 181: 3942–3948.

Wang LF, Wong SL, Lee SG, Kalyan NK, Hung PP, Hilliker S and Doi RH. 1988. Expression and se-

cretion of human atrial natriuretic a-factor in *Bacillus subtilis* using the subtilisin signal peptide. Gene 69: 39–47.

WIEGERT T, HOMUTH G, VESTEEG S AND SHUMANN W. 2001. Alkaline shock induces the *Bacillus subtilis* sigma (w) regulon. Mol Microbiol 41: 59–71.

WONG SL. 1995. Advances in the use of *Bacillus subtilis* for the expression and secretion of heterologous proteins. Curr Op Biotech 6: 517–522.

WU SC AND WONG SL. 1999. Development of improved pUB110-based vectors for expression and secretion studies in *Bacillus subtilis*. J Biotechnol 72: 185–195.

WU SC, YEUNG JC, DUAN Y, YE R, SZARKA SJ, HABIBI HR AND WONG SL. 2002. Funtional production and characterization of a fibrin-specific single chain antibody fragment from *Bacillus subtilis*: effects of molecular chaperones and a wall-bound protease on antibody fragment production. Appl Environ Microbiol 68: 3261–3269.

WU XC, LEE W, TRAN L AND WONG SL. 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol 173: 4952–4958.