Construction and characterisation of O139 cholera vaccine candidates

Talena Ledón a,*, Edgar Valle a, Tania Valmaseda b, Barbara Cedré b, Javier Campos a, Boris L. Rodríguez a, Karen Marrero a, Hilda García b, Luis García b, Rafael Fando a

a Grupo de Genética, Centro Nacional de Investigaciones Científicas, AP 6412 Havana, Cuba
b Instituto Finlay, Sueros y Vacunas, Havana, Cuba

Received 14 June 2001; received in revised form 25 March 2002; accepted 7 August 2002

Abstract

The hemagglutinin/protease (HA/P) seems to be an attractive locus for the insertion of heterologous tags in live cholera vaccine strains. A ΔCTXΦ spontaneous mutant derived from a pathogenic strain of O139 Vibrio cholerae was sequentially manipulated to obtain hapA::celA derivatives which were later improved in their environmental safety by means of a thyA mutation. All the strains here obtained showed similar phenotypes in traits known to be remarkable for live cholera vaccines irrespective of their motility phenotypes, although the hapA mutants had a 10-fold decrease in their colonisation capacity compared with their parental strains in the infant mouse cholera model. However, the subsequent thyA mutation did not affect their colonisation properties in the same model. These preliminary results pave the way for further clinical assays to confirm the possibilities of these vaccine prototypes as safe and effective tools for the prevention of O139 cholera.

© 2002 Published by Elsevier Science Ltd.

Keywords: O139 cholera vaccine; hapA mutants; thyA mutants

1. Introduction

Cholera remains a health threat in many of the developing countries. Before 1992, cholera was exclusively associated with Vibrio cholerae of the O1 serogroup. However, in late 1992 a new serogroup of the etiologic agent of cholera was identified in India [1]. The serogroup associated with this new epidemic was designated O139. Pre-existent immunity to O1 V. cholerae is not protective against vibrios of the O139 serogroup. Recent experiences have reinforced the need for effective cholera vaccines assuming that V. cholerae O139, currently confined to south-east Asia, can spread to other continents.

Because convalescence to cholera results in long-lasting protective immunity, much of the efforts in V. cholerae vaccinology have been made to produce live attenuated cholera vaccines that stimulate the mucosal immune system by mimicking natural infection [2]. Currently, several genetically engineered O1 El Tor and O139 candidate vaccine strains have been claimed to be well tolerated and immunogenic in volunteer studies [2–5]. The efficacy of these vaccine prototypes remains to be confirmed by field trials in endemic areas.

The soluble hemagglutinin/protease (HA/P) of V. cholerae [6] is a member of a family of zinc-dependent metalloproteases [7]. It proteolytically activates cholera toxin A subunit [8] and hydrolyses several physiologically important proteins, such as mucin, fibronectin, and lactoferrin [9]. It also inactivates the filamentous phage CTXΦ, which carries the genes for known V. cholerae enterotoxins [10]. Robert et al. [11] demonstrated the convenience of using the hemagglutinin/protease locus for the insertion of heterologous tags like the celA gene from Clostridium thermocellum, a suitable marker to clearly distinguish a O1 cholera vaccine strain from wild type V. cholerae. The celA gene from C. thermocellum encodes a thermophilic β(1–4) glucanohydrolase denoted as endoglucanase A (CelA) [12]. Benítez et al. have recently demonstrated that strain V. cholerae 638 possessing an insertional inactivation of hapA resulted a well-tolerated cholera vaccine candidate in healthy volunteers [4]. In contrast, the parental strain V. cholerae 81 induced untoward effects when was similarly tested (unpublished results).

Just like with live attenuated vaccines for other diseases, there is a potential safety issue for live cholera vaccines that is not valid for killed or subunit vaccines. Viable bacterial vaccine strains could revert to virulence; nevertheless, efforts have been made to diminish the possibility of this event. We have recently documented the usefulness of an internal deletion in the thyA gene of O1 V. cholerae vaccine candidates to enhance the environmental safety features.
without a detrimental effect on their colonising and immunising properties in animal models [13].

In the pathogenesis of cholera, bacterial colonisation of the human intestine is crucial to prime the mucosal immune system and to induce a strong secretory IgA response [14,15]. Colonisation of the gastrointestinal tract of infant mice is widely accepted to correlate well with colonisation of the human gut [14].

In this paper, we describe the construction and characterisation of different O139 cholera vaccine candidates derived from the wild type strain SG25-1, which cover a range of desirable features for cholera vaccines from an isogenic line. A ΔCTXΦ spontaneous mutant is first presented as SG25-1a. Two hapA::celA mutant derivatives of SG25-1a are then disclosed, one of which is also a non-motile spontaneous mutant. Finally, a thyA mutant of each hapA::celA derivative is likewise presented. In addition, we evaluated the effects of mutations in the hapA and thyA loci in the in vivo mouse colonisation model of cholera for our O139 V. cholerae vaccine candidates and their immunogenic potential in a rabbit model.

2. Materials and methods

2.1. Strains and media

Bacterial strains and plasmids used in this study are described in Table 1. All strains were grown in Luria–Bertani medium (LB) or tryptic soy broth and conserved at −70 °C in LB supplemented with 20% of glycerol. For in vitro toxin production, V. cholerae was streaked in blood agar plates and subsequently cultured by the AKI procedure [22]. Suicide vectors were propagated in E. coli SY327pir and mobilised from E. coli SM10pir into V. cholerae by conjugation. Antibiotics were added at the following concentrations (in µg/ml): ampicillin (Amp), 100; chloramphenicol, 100; trimethoprim, 50; polymyxin B, 34; and sodium pyruvate, 1.32. Thymidine (Thy) was used at 200 or 50 µg/ml when necessary.

2.2. Plasmid construction

V. cholerae chromosomal DNA was prepared as described by Ausubel et al. [16]. Plasmid DNA minipreparations were performed as described by Birnbom and Doly [23]. DNA restriction and modification enzymes were purchased from Boehringer Mannheim and used according to manufacturer’s instructions. Recombinant plasmids were constructed using standard methods [24]. The suicide plasmid pGPH6, used to obtain the hapA::celA mutants of strain SG25-1a, was previously described [11]. The suicide plasmid pCVTAT (Table 1) was constructed in several steps. First, a mutant allele of thyA bearing a deletion at the S′ end of thyA was cloned from pBMTA (Table 1) as a SphI–HindIII fragment into Smal–HindIII-digested pUC19 to obtain pUTAT. This construction rendered the thyA-mutated allele flanked by SacI restriction sites. Finally, the thyA allele was excised as a SacI fragment from pUTAT and inserted into equally digested pCVD442.

2.3. Strain construction

V. cholerae O139 strain SG25-1 (Table 1) was used as the initial progenitor for constructing three generations of vaccine prototypes. The genotype of each prototype was tested in Southern blot after alkaline capillary transfer of DNA to nitrocellulose filters [25] and detection was performed with probes generated from the following fragments: the ctxA probe consisted of a 643 bp fragment amplified by PCR using the primer pairs 5′-AGATCTCGACGTAAGAACTC-3′ (sense) and 5′-AGGTTGTTCCATGTCGTTATGC-3′ (antisense), the 2.9 kb EcoRI–PstI fragment containing the RS1 element from plasmid pURSI [21] was used as the RS specific probe and the HindIII insert of pCH2 [20] was used as a hapA specific probe. Finally, the 1.4 kb EcoRI–HindIII thyA fragment internal to the insert in pVTa [13] was used as the thyA specific probe. All the probes were generated using the DNA labelling and detection kit of Boehringer Mannheim. The strategy for constructing cholera vaccine prototypes here analysed is shown in Fig. 1. The first generation (ΔCTXΦ) resulted from a spontaneous deletion event of CTXΦ genomes in strain SG25-1, thus producing strain SG25-1a. This strain was later engineered using a previously described methodology of marker exchange [11] with plasmid pGPH6 to obtain HA/P defective, CelA expressing O139 cholera vaccine candidates.

Defined mutants, deficient in thymidilate synthase biosynthesis were constructed by allelic replacement of the thyA gene in chromosome I of HA/P defective strains with the mutated allele in plasmid pCVTAT (Table 1), which was mobilised from SM10pir into V. cholerae by filter mating. Exconjugants were selected in LB plates supplemented with ampicillin and polymixin B and correct co-integrates, originated from the integration of plasmid sequences in the thyA locus by homologous recombination, were screened by means of Southern blots. One of such correct co-integrates from each hap mutant was cultured overnight in LB broth supplemented with thymidine (200 µg/ml) and culture dilution aliquots were plated in LB prepared with omission of NaCl and supplemented with 15% sucrose and thymidine (200 µg/ml). Under these conditions, the plasmid-encoded sacB allowed positive selection of bacteria in which a second recombinational event deleted vector DNA from the chromosome. Several of these colonies were tested for the presence of the desired allele. Derivatives carrying the mutated allele were conserved for future studies.

2.4. Phenotypic characterisation

2.4.1. Determination of cholera toxin (CT) production

CT was determined using a GM1 ganglioside-dependent enzyme-linked immunosorbent assay (ELISA) [26].
Table 1  
Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>supE44 ΔlacZΔM15ΔlacI857 recA1 endA1 gyrA96 thi-1 mcrA</em></td>
<td>[16]</td>
</tr>
<tr>
<td>SY327 (pir)</td>
<td>Δlac proE argE1(ami) rifA endA1 recA1860 (proBR6K), host for suicide vectors</td>
<td>[17]</td>
</tr>
<tr>
<td>SM010 (pir)</td>
<td><em>thyA leu thi F supE recA387 RP4-2Tc-Mu</em> (Hf8084), Km r, host for suicide vectors with transfer functions integrated in the chromosome</td>
<td>[18]</td>
</tr>
<tr>
<td>V. cholerae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SG25-1</td>
<td>Wild type, O139, Calcutta, India, 1993</td>
<td>R. A. Finkelstein</td>
</tr>
<tr>
<td>SG25-1a</td>
<td>spontaneous ΔCTXΦ mutant from SG25-1</td>
<td>This study</td>
</tr>
<tr>
<td>81</td>
<td>ΔCTXΦ mutant from wild type C7258</td>
<td>[19]</td>
</tr>
<tr>
<td>838</td>
<td>hapAΔΔ::HindIII mutant from 81</td>
<td>[11]</td>
</tr>
<tr>
<td>L911</td>
<td>SG25-1a, hapAΔΔ, spont. non-mobile mutant</td>
<td>This study</td>
</tr>
<tr>
<td>L912</td>
<td>SG25-1a, hapAΔΔ</td>
<td>This study</td>
</tr>
<tr>
<td>L911T</td>
<td>thyA mutant of L911</td>
<td>This study</td>
</tr>
<tr>
<td>L912T</td>
<td>thyA mutant of L912</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGPH6</td>
<td>The HindIII fragment from <em>V. cholerae</em> 3083 coding for HA/P after insertion inactivation of <em>hapA</em> cloned as a BglII–HindIII insert in pGP704, ampR</td>
<td>[11]</td>
</tr>
<tr>
<td>pGPH7</td>
<td>HapA as a HindIII fragment of 3.2 kb from <em>V. cholerae</em> 3083 cloned into pACYC184, chloro</td>
<td>[20]</td>
</tr>
<tr>
<td>pVT1</td>
<td>thyA as a BamHI–HindIII fragment of chromosomal DNA from <em>V. cholerae</em> C7258 cloned into pBR322, ampR</td>
<td>[13]</td>
</tr>
<tr>
<td>pBMTA</td>
<td>A BamHI–HindIII fragment, carrying thyA from <em>V. cholerae</em> C7258 with a MfeI–BglII internal deletion cloned into pBR322, ampR</td>
<td>This study</td>
</tr>
<tr>
<td>pUTAT</td>
<td>An SstI–HindIII fragment with the mutated thyA from pBMTA cloned at <em>SalI–HindIII</em> sites of pUC19, ampR</td>
<td>This study</td>
</tr>
<tr>
<td>pCVTAT</td>
<td>The SstI fragment, carrying the mutated thyA, from pUTAT cloned into pCVD442, ampR</td>
<td>This study</td>
</tr>
<tr>
<td>pURS1</td>
<td>The RS1 fragment amplified from <em>V. cholerae</em> 81 cloned in the <em>SmaI</em> site of pUC19, ampR</td>
<td>[21]</td>
</tr>
</tbody>
</table>

Fig. 1: Schematic diagram of the construction of *V. cholerae* O139 vaccine candidates derived from the spontaneous CTXΦ deletion mutant SG25-1a. Arrows indicate steps of co-integrate formation or resolution at different loci of the *V. cholerae* genome. The gene targeted for each manipulation appears indicated, as well as the suicide plasmid used in each step. Mutant genes are represented as *thxA* and *hapA*. Dashed lines represent the recombination event and relevant restriction sites are also indicated.
Monoclonal antibodies (Mabs) 1G10G5 and 4E1G5 directed against cholera toxin subunits CTa and CTb, respectively, were used as primary antibody and peroxidase-conjugated anti-mouse IgG (whole molecule, Sigma) as secondary antibody.

2.4.2. Detection of HA/P and mannose-sensitive hemagglutinin (MSHA)

Gel electrophoresis and Western blot analysis were performed as previously described [27]. Samples were prepared from 50 ml of tryptic soy broth (TSB) cultures in 500 ml bottles inoculated with fresh colonies after overnight growth. V. cholerae supernatants were concentrated 5–10-fold in Centricon-10 microconcentrators and fractionated by SDS-PAGE according to Laemmli [28]. HA/P protein bands were detected as previously described [20] with a polyclonal rabbit antiserum raised against purified HA/P kindly provided by R.A. Finkelstein. MSHA bands in Western blots of whole cell lysates were detected using a specific monoclonal antibody [29].

2.4.3. Endoglucanase assay

Endoglucanase A activity in V. cholerae was detected by overlaying LB agar grown colonies on plates with CMC-indicator agar (0.7% agarose, 0.5% CM-cellulose in phosphate–citrate buffer pH 6.3), incubating 2 h at 60 °C. Positive colonies were visualised as red colonies surrounded by a transparent halo in the red background of the plate.

2.4.4. Hemagglutination

Microtitre quantification of hemagglutinating activity was determined with 1% (v/v) chicken red blood cells in microtitre plates. Cell associated and soluble hemagglutination (HA) and hemagglutination inhibition (HAI) tests were also performed as previously described [30]. The titre was defined as the reciprocal of the highest bacterial or protein dilution that caused hemagglutination.

2.4.5. Assay for motility

Isolated colonies were picked from a master plate and inoculated by insertion (2–3 mm) into motility agar plates. Next, one volume of a 10^6 CFU/ml suspension of V. cholerae colonies were also examined for motility by light microscopy.

2.4.6. Morphology and antibiotic resistance

Cell morphology was evaluated by light microscopy and the presence of capsule was verified as described by others [31]. Briefly, V. cholerae strains were grown for 3 h at 37 °C on LB broth and bacteria were negatively stained with 1% (w/v) uranyl acetate for 3 min, and analysed by transmission electron microscopy. Bacterial strains were also tested for their susceptibility to streptomycin at 100 μg/ml on LB plates and to sulfamethoxazole (23.75 μg) and trimethoprim (1.25 μg) (SXT) by the disc diffusion technique [32].

2.5. Assay for protease activity

Protease activity in supernatants of TSB-cultured vibrios was qualitatively detected by single radial diffusion in a 1.5% agar gel containing 1.5% skim milk as substrate. The activity was more accurately quantified using the azocasein assay adapted from [33]. Briefly, 1.1 ml of buffer (CaCl_2, 1 mM; Tris–HCl, 0.2 M; pH 7.2; azocasein, 1%) was mixed with 200 μl of culture supernatants and incubated for 1 h at 37 °C. The unreacted substrate was precipitated with 85 μl of 40% TCA for 10 min, followed by 10 min of centrifugation at 12,000 rpm. The coloured product remaining in solution was neutralised with NaOH and read at 450 nm. One unit of enzymatic activity was defined as the quantity of enzyme producing a net increase of one in the optical density of the sample in 1 h of reaction.

2.6. Intestinal colonisation assay

The infant mouse colonisation assay [14] was used to assess the colonisation properties of each strain. The inoculum, consisting of 10^8–10^9 vibrios in a final volume of 50 μl, was intragastrically administered to groups of at least five 3–5-day-old BALB/c mice. Infant mice were caged separately from their mothers and after 18–24 h, the small intestine was removed, homogenised and droplets of 10-fold serial dilutions of the bacterial suspensions were plated on appropriate media for bacterial counting.

2.7. Immunological analysis

The immunising potential of mutant strains in the adult rabbit cholera model was analysed. At least three New Zealand rabbits for each strain, weighing 1.1–1.2 kg, were fasted overnight and intraduodenally inoculated with about 1 × 10^8 CFU. Blood samples were taken at days 0, 7, 14, 21 and 28, stored at −80 °C and later examined for the presence of anti O139 LPS IgG antibodies in the serum essentially as described [4]. Vibriocidal antibody titres were also determined in a microassay (14). Briefly, two-fold serial dilutions of sera in saline (25 μl) were placed in 96-well tissue culture plates. Next, one volume of a 10^7 CFU/ml suspension of V. cholerae O139 SC225 or MO45, containing undiluted human complement without anticholera activity was added to each well and incubated for 1 h at 37 °C. Finally, BHI broth containing 2% dextrose and 2% bromocresol purple was added and the plates were incubated 3 h at the same temperature. The vibriocidal antibody titre was defined as the highest dilution of serum causing complete inhibition of bacterial growth as judged by visual colour comparison of the culture medium with a control without serum. Because of reduced sensitivity to complement by O139 strains, complement was used at a final concentration
five times the concentration used for El Tor V. cholerae strains and less quantity of target bacteria was used.

3. Results

3.1. Construction of vaccine prototypes

Molecular genetic studies of V. cholerae O1 and O139 strains have revealed that the ctxAB genes encoding cholera toxin reside within the genome of a filamentous, lysogenic bacteriophage known as CTXφ [34]. In El Tor strains, prophage DNA is usually found in tandem arrays frequently associated with a related genetic element known as RS1 [35]. Our genetic studies with V. cholerae SG25-1 revealed that a relatively frequent spontaneous event leads to deletion of the CTXφ prophage sequences that leaves behind a single copy of the RS1 element. The presence of this RS1 element and the lack of ctxA genes in one of the clones termed SG25-1a, was characterised by Southern hybridisation with ctxA and RS specific probes. Southern blots of PstI-digested chromosomal DNA from SG25-1a, probed with the ctxA specific probe showed the absence of cross-hybridising material (Fig. 2A, lane b). In contrast, equally digested chromosomal DNA from SG25-1 produced a single ctxA specific band migrating between the 9 and 23 kb marker DNA fragments (Fig. 2A, lane a).

Additionally, Southern blots of EcoRV- or BglII-digested DNAs from SG25-1a, probed with the RS and ctxB specific probes produced a pattern similar to that of 638, an atoxicogenic derivative of El Tor strain C7258, which is known to have a single RS1 element remaining on its chromosome (Fig. 2B and data not shown). Finally, the analysis with probes specific for the rest of the CTXφ prophage genes (cep, orfU, ace, zot and ctxB) demonstrated their absence from SG25-1a genome (data not shown). Taken together, these results indicate that the deletion of CTXφ prophage genome occurred in SG25-1 left behind a single RS1. Strain SG25-1a was conserved as our first atoxicogenic O139 cholera vaccine candidate for further characterisation.

A second generation of cholera vaccine candidates was obtained after replacing the chromosomal hapA gene in strain SG25-1a with the hapA::celA allele present in vector pGPH6 (Table 1). This process, passing through an AmpR co-integrates forming step, yielded several clones of SG25-1a, in which hapA had been replaced by hapA::celA. Two of these clones were selected and denominated L911 and L912. Southern blot hybridisation analysis of XhoI digested DNAs from SG25-1a, the AmpR co-integrates, L911 and L912, were performed. The results presented in Fig. 2C confirmed the expected hybridisation pattern with regards to motility. We found reasonable constructivity with the expected specific fragment in the co-integrates consistent with the insertion of 3.2 kb of celA DNA into the XhoI hapA-specific fragment in strain SG25-1a.

Fig. 2. Southern blot hybridisation analysis of wild type V. cholerae SG25-1 and derived O139 vaccine candidates. V. cholerae 638 was compared as a control. Chromosomal DNAs were appropriately digested and probed with: (A) the ctxA specific probe; (B) the RS1 specific probe; (C) the hapA specific probe; (D) the thyA specific probe. In (A), chromosomal DNAs from SG25-1 (lane a) and SG25-1a (lane b) were digested with PstI. In (B), chromosomal DNA from 638 was digested with EcoRV (lane a) or BglII (lane c), and chromosomal DNA from SG25-1a was digested with EcoRV (lane b) or BglII (lane d). In (C), DNA from all strains was digested with XhoI, lane a, SG25-1a; lane b, co-integrate of SG25-1a with pGPH6; lane c, L911; lane d, other co-integrate of SG25-1a with pGPH6, lane e, L912. In (D), chromosomal DNAs were double digested with CleI and XhoI and placed in the following order: lane a, L911; lane b, co-integrate of L911 with pCVTA T; lane c, L911T; lane d, co-integrate of L912 with pCVTA T; lane e, L912T. Bands of the molecular weight marker are indicated in kb.
described in Section 2 was used. Fig. 2D shows a Southern blot of Clal--Stul double digests of chromosomal DNA from the thyA mutant vaccine prototypes obtained (L911T and L912T), probed with the thyA specific probe (see Section 2). As neither Clal, nor Stul cut the locus for thyA, a single band hybridising with thyA was expected from L911, L912 and the corresponding derivatives, except from the co-integrates because Clal sites are present in pCVTA T. Evident differences of 300 bp were seen in the length of fragments from progenitors L911 or L912 and the thyA mutants L911T or L912T, consistent with the deletion performed in the thyA gene to obtain pCVTA T.

3.2. Phenotypic characterisation

V. cholerae vaccine constructs SG25-1a, L911, L912, L911T or L912T were serogrouped as O139 with a specific antiserum and did not produce detectable levels of CT when assayed by GM1-ELISA (detection limit 0.1 ng/ml), in contrast to SG25-1. Strains L911, L912, L911T and L912T lacked soluble hemagglutinating activity and cross-reacting bands in Western blots with an anti HA/P polyclonal antiserum and did not produce detectable levels of CT when assayed by GM1-ELISA (detection limit 0.1 ng/ml), in contrast to SG25-1. Strains L911, L912, L911T and L912T also showed reduced extracellular proteolytic activity compared with the parent strain SG25-1a, the expected 17 kDa band in all strains (not shown).

To determine whether successive genetic manipulations had unexpected effects over other important characteristics of cholera vaccine candidates, we examined their growth rate and expression of several important antigens. Strains SG25-1, SG25-1a, L911 and L912 grew with the same kinetics and exhibited doubling times of 20 ± 2 min in rich broth. The mean generation time of L911T and L912T, growing in LB supplemented with thymidine at 50 μg/ml was indistinguishable from that of L911 and L912, respectively.

Strains SG25-1, SG25-1a, L912 growing in LB broth or strain L912T growing in thymidine (200 μg/ml) supplemented LB broth, exhibited normal rod shaped cells when examined at the optical microscope. In contrast, strain L911 and its auxotrophic derivative L911T, exhibited a filamentous phenotype, when growing in LB or thymidine supplemented LB broth, respectively; although in L911T this abnormal morphology appeared in less degree and number at the same optical density at 600 nm. The filamentous phenotype of these vaccine strains showed a time-course dependency during growth. The number of elongated cells raised proportionally to the increase in optical density at 600 nm until a point when they started to decrease. This point was not exactly determined but in stationary phase cultures almost all the cells had become normal.

When appropriate samples were taken at late stationary phase for light microscopy examination, we observed that cholera bacilli of thyA mutants showed an elongated phenotype, whose number increased proportionally to the thymidine limitation.

Strains SG25-1, SG25-1a, L912 and L912T were found to be motile in motility agar plates, while strains L911 and L911T were demonstrated to be non-motile. Strains L911 and L911T were flagellated, indicating that the lack of flagella is not the cause of the non-motile phenotype. The filamentous phenotype of strain L911 seemed not to be the unique cause of non-motility since L912T continued to be motile (although in less proportion) when grown in thymidine-limiting conditions where the filamentous phenotype was observed. This non-motile phenotype was not associated with reversion to motility with successive passing, unlike Peru 15 [5], it was stably inherited in both strains.

O139 vaccine candidates were tested for cell associated HA and in HAI assays. All of them were hemagglutinative and sensitive to inhibition by mannose. According to this, MSHA expression was investigated by immunoblot of whole cell lysates. The anti MSHA MAb 2F12F1 [29] recognised the expected 17 kDa band in all strains (not shown).

Capsule production was also verified by electron microscopy as well as other features that differentiate O139 from strains belonging to the El Tor biotype of O1 vibrios, like sensitivity to SXT [37]. A self-transmissible, site-specific 62 kb conjugative transposon encodes the functions that confer SXT and streptomycin resistances to O139 strains [38]. The susceptibility of SG25-1 and its derivatives to these antibiotics were tested and all of them were confirmed to be resistant to SXT and streptomycin.
1.3. Protease activity

The protease activity of strains used in this work and other El Tor strains manipulated in our laboratory was accurately quantified using azocasein assay (Table 2). The mutation introduced in the hemagglutinin/protease gene accounts for a reduction in 80–85% of proteolytic activity as observed in mutants of both serogroups when compared to their non-toxigenic parents. Nevertheless, the mutant strains still produced some extracellular proteolytic activity, which could be explained by several other proteases present in V. cholerae [39].

3.4. Colonisation properties

The ability of mutant strains of V. cholerae to colonise the intestine of suckling mice can be observed in Table 3. No statistical difference could be detected between hapA mutants and their thyA derivatives or between SG25-1a and its virulent parent. Although all of them retained the ability to colonise the infant mice small bowel, as shown in Table 3, it should be noted that strain L911 and L912, lacking HA/P, colonised less in this animal model than their parental strain SG25-1a. They showed a 10-fold decrease in the colonisation capacity. L911 colonised the small bowel of infant mice like L912; thus, the filamentous phenotype had no influence on its colonisation properties. L911, L912, L911T and L912T vibrios isolated from mice intestines were also found to express an endoglucanase A positive phenotype (not shown).

Table 4: Immune response to L911, L912, L911T and L912T in rabbits intraduodenally inoculated with a single dose of 10^7 CFU of each vaccine candidate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vibriocidal antibodies</th>
<th>Anti-LPS IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td>Day 28</td>
<td>Day 14</td>
</tr>
<tr>
<td>L911</td>
<td>127 (80–160)</td>
<td>160 (160)</td>
</tr>
<tr>
<td>L912</td>
<td>177 (80–160)</td>
<td>216 (40–160)</td>
</tr>
<tr>
<td>L911T</td>
<td>320 (80–1280)</td>
<td>127 (20–640)</td>
</tr>
<tr>
<td>L912T</td>
<td>80 (40–160)</td>
<td>127 (80–520)</td>
</tr>
</tbody>
</table>

Day 0 titres were under 1:20 for vibriocidal antibodies and under 1:25 for anti-LPS IgG.

4. Discussion

The CTX prophage–RS1 arrays differ widely among pathogenic O1 and O139 by both, the number and relative
arrangement. Numerous patterns have been detected in O1 El Tor strains as well as in O139 strains [40–43]. Experiments to determine the genetic organisation of these genes and mechanisms by which the toxigenic strain SG25-1 gave rise to the attenuated strain SG25-1a are in progress. However, recombination between RS elements and/or other uncommon events can not be disregarded. Several findings have indicated that even in strains of clonal origin, the CTXΦ prophage genome can undergo rearrangements leading to amplifications or deletions [40,42]. Important questions concerning the evolution of O139 strains remain and the elements that will emerge from studying the organisation of CTXΦ in SG25-1 will constitute additional information.

Live oral attenuated vaccines offer great promise for preventing cholera because a single dose elicits high-titers of serum vibriocidal antibodies, the best known immunological correlate of protection [44]. The rapid spread of V. cholerae O139 among all ages in areas where O1 cholera is epidemic indicates that serotype-specific vaccines are needed. The experience gained from construction of attenuated O1 vaccine prototypes [5,19] provide the basis for the development of live attenuated vaccine candidates for the prevention of cholera due to V. cholerae O139.

The attenuated strain SG25-1a, a spontaneous derivative of O139 wild type SG25-1, devoid of CTXΦ prophage sequences and that retained a single RS1 copy in the chromosome was used to develop other prototypes with better vaccine attributes.

The presence of the RS1 element in the genome of the vaccine prototype SG25-1a could be controversial considering the possibility of reacquisition of CTXΦ. The SG25-1a DNA restriction analysis showed that this strain harbours an El Tor-type CTXΦ prophage, the most prevalent nowadays. This element suggests that El Tor rsRΦ can be a desired attribute to protect this live vaccine strain from infection with the El Tor CTXΦ and thereby significantly lowers the possibility of vaccine reversion to toxigenicity [45]. However, these strains remain sensitive to classical and O139 (Calcutta) phages. Thus, additional mutations are desired to prevent or make superfluous reacquisition of CT genes.

Like for some of our O1 cholera vaccine candidates, the newly constructed O139 candidates were marked by the insertion of celA, a gene conferring endoglucanase A activity, into the hemagglutinin/protease locus. The resulting strains termed L911 and L912, possesses the following traits: (1) they are devoid of CT expression; (2) they lack detectable soluble hemagglutinating activity; and (3) they can be unequivocally distinguished by their cellulolytic activity. CelA has demonstrated to be an excellent marker to our vaccine strains to lysis by antibody and complement, and that other components like complement and indicator bacteria concentration, as well as the assay conditions readily compromised detection of antibacterial responses. In our experiments we adjusted only the number of cells and the concentration of the complement. Although no other suggestions to improve the technique were taken into account, the results seem to be consistent.

The non-motile mutant L911 was not defective in intestinal colonisation of suckling mice in regard to the motile variant L912 (Table 4). This finding parallels the experience with motility deficient El Tor O1 strains, such as Peru 14, non-motile Peru 15 and the O139 strain Bengal 15 [3,5].

Several hypotheses have been proposed to explain the reagogenic properties of attenuated vaccines: one of the most convincing deals with the role of motility in residual symptoms seen in volunteers ingesting attenuated live cholera vaccines. Peru 14, Peru 15 and Bengal 15 were non-reactogenic but still elicited significant and protective immune responses [3,5]. According to the “mucus gel penetration = reagogenicity” hypothesis [48], one would expect that L911 turned to be well tolerated. On the other hand, we reasoned that our strategy directed to the inactivation of the major secreted protease (80–85% of soluble protease activity), responsible for mucin degradation [9], would render strains with limited capacity to reach the enterocytes and, therefore, reduce their ability to directly stimulate them to release cytokines, which can promote an inflammatory response. There are other reasons to consider HA/P as a
prominent candidate for a reaginic factor. Wu et al. have shown that HA/P can act as a cytotoxin that perturbs the barrier function of epithelial cells (MDCK-I) by changing their morphology, reorganising the tight junction-associated protein ZO-1 and the F-actin cytoskeleton and degrading another physiologically important protein [49]. More recently, MeL et al. [50] have found that culture supernatants from strains with hapA protease gene deleted, showed little effect on the transcellular epithelial resistance of T84 intestinal cells. Furthermore, previous experiences of our group with the El Tor vaccine prototype 638, a hapA mutant, showed a well-tolerated phenotype and elicited significant immune responses when tested in volunteers [4]. This strain also displayed a reduced IL-8 response in the undifferentiated intestinal cell line HT29-18N2, similar to the non-reagentic strain CVD103HgR [51]. Then, the combination of Mot+ and Hap+ phenotypes could result profitable to L911 in regard to its properties as a vaccine candidate.

Production of a safe vaccine against cholera has been cumbersome due to residual reagenticity. We consider that the overall pathogenesis of V. cholerae is a complex issue and more investigation is needed to understand the exact role of HAP in the physiology and pathogenesis of this infection.

The importance of thyA for colonisation has been the subject of somewhat discrepant conclusions. Early studies with V. cholerae CVD102, a spontaneous thymidine auxotroph of CVD101, a vaccine candidate generated from classical strain O139, exhibited a vibriocidal response of very low titre and no anti-toxin seroconversion in volunteers, indicating that the auxotrophic mutation was overattenuating [52]. More recently other researchers found that there was a previously unrecognised mutation responsible for the poor in vivo performance of CVD102, probably associated with the reduced synthesis of toxin co-regulated pilus (TCP) during in vitro and in vivo growth [53]. Additionally another spontaneous mutant selected by trimethoprim resistance, 815, was also defective for colonisation of the small bowel of mice when compared to its progenitor strain 81 [11]. Results obtained in this study and other previously performed experiments with El Tor strain 638T [13] allowed us to conclude that an intact thyA gene is not essential for colonisation of the intestine of suckling mice or rabbits while being an essential enzyme for the environmental survival of V. cholerae O1.

So, it is useful to our purpose of limiting the ability of vaccine prototypes to survive in environmental reservoirs if we render biologically contained live cholera vaccines by means of thyA inactivation. In these cholera vaccines the acquisition of CTXf phase-ge phenotype is superfluous. Tests in volunteers should be done to confirm the features of these candidates regarding colonisation and immunogenicity.

The results described above have motivated us to propose hapA and thyA defined mutations, in addition to CTXf deletions, as a suitable combination to produce a protective O139 vaccine, with enough safety for human use and environmental release.

Acknowledgements

We thank Arlenis Moreno for invaluable technical assistance in the preservation and maintenance of bacterial strains. We are indebted to R.A. Finkelstein for providing V. cholerae strain SG25-1 and anti-HA/P antisera. We also recognise to Odessa Ancheta her help in electron microscopy analysis of our V. cholerae strains. Special thanks to Yussuan Silva and Emilio Marrero for assistance with graphics and animal models, respectively, and Professor Jesús Nuñez for his thorough revision of this manuscript.

References


