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Construction and characterisation of O139 cholera vaccine candidates

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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.
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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]. Recently, 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 proteolitically 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

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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 infant 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 *Escherichia coli* SY327λpir and mobilised from *E. coli* SM10λpir into *V. cholerae* by conjugation. Antibiotics were added at the following concentrations (in μg/ml): ampicillin (Amp), 100; chloramphenicol, 34; and polymyxin B, 13.2. 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 Birnboim 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 5′ end of *thyA* was cloned from pBMTA (Table 1) as a *Ssp*I–*Hin*dIII fragment into *Sma*I–*Hin*dIII-digested pUC19 to obtain pUTAT. This construction rendered the *thyA*-mutated allele flanked by *Sac*I restriction sites. Finally, the *thyA* allele was excised as a *Sac*I 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-ATGATCATGCAAGAGGAACTC-3 (sense) and 5-AGGTGTTCCATGTGCATATGC-3 (antisense), the 2.9 kb *Eco*RI–*Pst*I fragment containing the RS1 element from plasmid pURS1 [21] was used as the RS specific probe and the *Hin*dIII insert of pCH2 [20] was used as a *hapA* specific probe. Finally, the 1.4 kb *Eco*RI–*Hin*dIII *thyA* fragment internal to the insert in pVTα [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 SM10λpir into *V. cholerae* by filter mating. Exconjugants were selected in LB plates supplemented with ampicillin and polimixin 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 immunoabsorbent assay (ELISA) [26].

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
DH5α	*supE44 Δlac(IZYA)U169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[16]
SY327λpir	*Δ(lac pro) argE*(Am) *rif nalA recA56* (λ*pirR6K*), host for suicide vectors	[17]
SM10λpir	*thi thr leu tonA lacY supE recA*::*RP4-2Tc*::*Mu* (λpirR6K), Km^r, host for suicide vectors with transfer functions integrated in the chromosome	[18]
V. cholerae		
SG25-1	Wild type, O139, Calcutta, India, 1993	R.A. Finkelstein
SG25-1a	Spontaneous ΔCTXΦ mutant from SG25-1	This study
81	ΔCTXΦ mutant from wild type C7258	[19]
638	*hapA*::*celA* mutant from 81	[11]
L911	SG25-1a, *hapA*::*celA*, spontaneous non-motile mutant	This study
L912	SG25-1a, *hapA*::*celA*	This study
L911T	*thyA* mutant of L911	This study
L912T	*thyA* mutant of L912	This study
Plasmid		
pGPH6	The *Hin*dIII fragment from *V. cholerae* 3083 coding for HA/P after insertional inactivation of *hapA* with *celA*, cloned as a *Bgl*II ended insert in pGP704, amp^r	[11]
pCH$_2$	*hapA* as a *Hin*dIII fragment of 3.2 kb from *V. cholerae* 3083 cloned into pACYC184, clm^r	[20]
pVTα	*thyA* as a *Bam*HI–*Hin*dIII fragment of chromosomal DNA from *V. cholerae* C7258 cloned into pBR322, amp^r	[13]
pBMTA	A *Bam*HI–*Hin*dIII fragment, carrying *thyA* from *V. cholerae* C7258 with a *Mlu*I–*Bgl*II internal deletion cloned into pBR322, amp^r	This study
pUTAT	An *Ssp*I–*Hin*dIII fragment with the mutated *thyA* from pBMTA cloned at *SmaI*–*Hin*dIII sites of pUC19, amp^r	This study
pCVTAT	The *Sac*I fragment, carrying the mutated *thyA*, from pUTAT cloned into pCVD442, amp^r	This study
pURS1	The RS1 fragment amplified from *V. cholerae* 81 cloned in the *Sma*I site of pUC19, amp^r	[21]

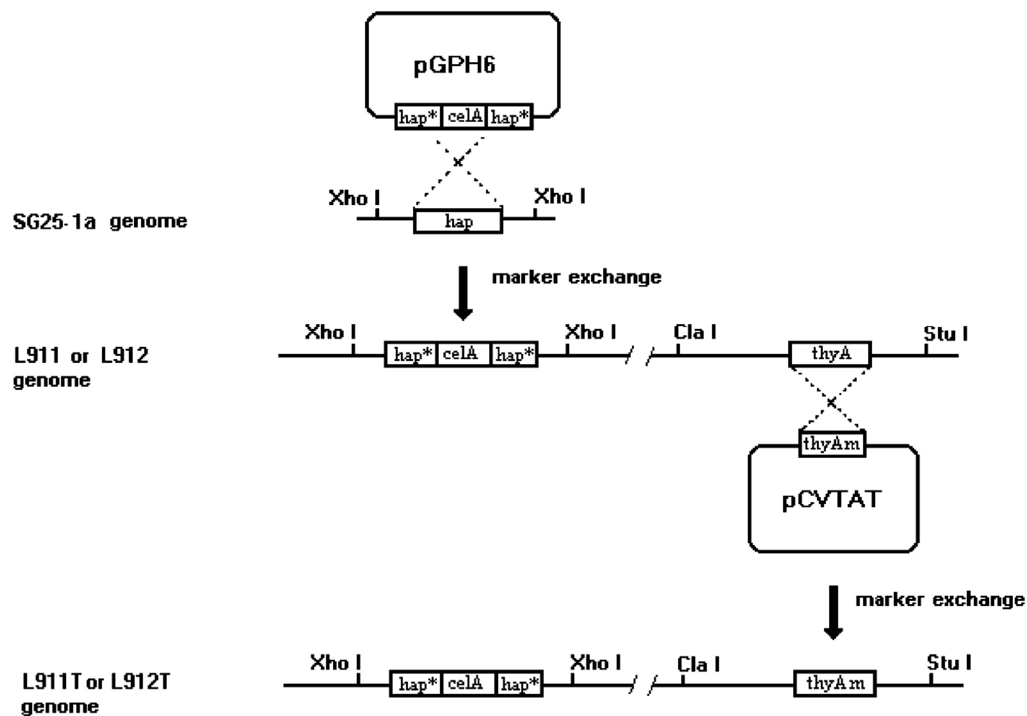

Fig. 1. Schematic diagram of the construction of *V. cholerae* O139 vaccine candidates derived from the spontaneous CTXΦ deletion mutant SG25-1a. Arrows indicate steps of co-integrate formation or resolution at different loci of the *V. cholerae* genome. The gene targeted for each manipulation appears indicated, as well as the suicide plasmid used in each step. Mutant genes are represented as $thyA_m$ and *hap**. 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, staining with 1% Congo red and destaining with 1 M NaCl. 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 (LB agar 0.4%). The diameter each colony spread through soft agar was recorded at 24 h of incubation at 30 °C. A bacterial strain spreading 3 mm or less from the point of application was considered non-motile. *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 10^6–10^7 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 1–1.2 kg, were fasted overnight and intraduodenally inoculated with about 1×10^9 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 10^6 CFU/ml suspension of *V. cholerae* O139 SG25 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 *Pst*I-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 *Eco*RV- or *Bgl*II-digested DNAs from SG25-1a, probed with the RS and *rstC* specific probes produced a pattern similar to that of 638, an atoxigenic 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 atoxigenic 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 Amp^r 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 *Xho*I digested DNAs from SG25-1a, the Amp^r co-integrates and their Amp^s segregates, L911 and L912, were performed. The results presented in Fig. 2C confirmed the expected genotypes. As neither *hapA*, nor *celA* or pGPH6 contain internal sites for the restriction enzyme *Xho*I, a single *hapA* specific fragment was detected in either SG25-1a, the co-integrates, L911 or L912. The size of the *hapA* specific fragment in the co-integrates was equivalent to the sum of

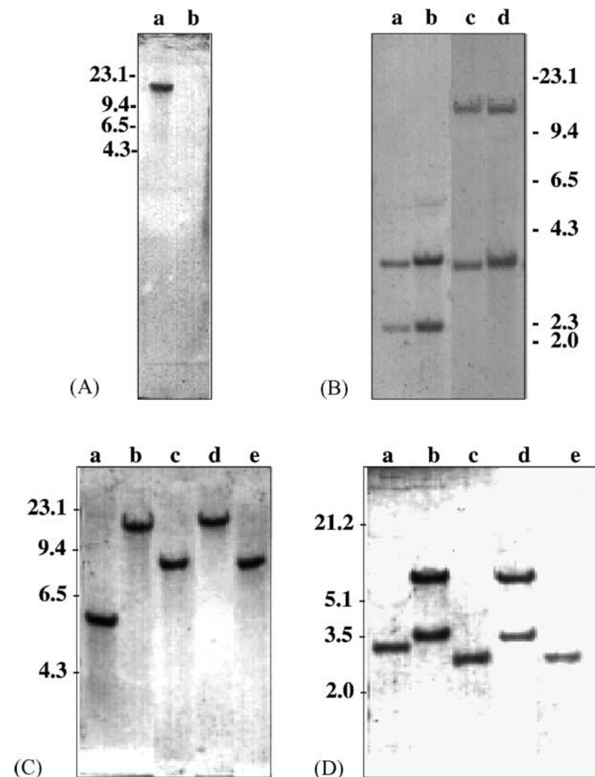

Fig. 2. Southern blot hybridisation analysis of wild type *V. cholerae* SG25-1 and derived O139 vaccine candidates; *V. cholerae* 638 was compared as a control. Chromosomal DNAs were appropriately digested and probed with: (A) the *ctxA* specific probe; (B) the RS1 specific probe; (C) the *hapA* specific probe; (D) the *thyA* specific probe. In (A), chromosomal DNAs from SG25-1 (lane a) and SG25-1a (lane b) were digested with *Pst*I. In (B), chromosomal DNA from 638 was digested with *Eco*RV (lane a) or *Bgl*II (lane c), and chromosomal DNA from SG25-1a was digested with *Eco*RV (lane b) or *Bgl*II (lane d). In (C), DNA from all strains was digested with *Xho*I: 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 *Cla*I and *Stu*I and placed in the following order: lane a, L911; lane b, co-integrate of L911 with pCVTAT; lane c, L911T; lane d, co-integrate of L912 with pCVTAT; lane e, L912T. Bands of the molecular weigh marker are indicated in kb.

the pGPH6 size plus the size of the *hapA* specific fragment in SG25-1a, consistent with the integration of pGPH6 into the *hapA* locus. In L911 and L912, the approximately 9 kb band hybridising with the *hapA* probe in lanes c and e is consistent with the insertion of 3.2 kb of *celA* DNA into the *Xho*I *hapA*-specific fragment of about 6 kb in the former SG25-1a. This band in the L911 and L912 *Xho*I digests also hybridised with the *celA* specific probe (data not shown). Taken together, these data demonstrated the replacement of *hapA* with the *hapA* : : *celA* allele from pGPH6 in SG251-a. Strains L911 and L912 were also conserved for further analysis.

As it will be discussed below, L911 and L912 are different with regards to motility. We found reasonable constructing *thyA* derivatives from both of them. The methodology

described in Section 2 was used. Fig. 2D shows a Southern blot of *Cla*I–*Stu*I 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 *Cla*I, nor *Stu*I 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 *Cla*I sites are present in pCVTAT. 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 pCVTAT.

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 antibody [36] (Fig. 3; lanes b and d), which reappeared in the mutants when transformed with pCH2 (Fig. 3; lanes c and e). L911, L912, L911T and L912T also showed reduced extracellular proteolytic activity compared with the parent strain SG25-1a as demonstrated on milk-containing agar plates and regained the ability to produce large clearing zones on milk agar when they were transformed with pCH_2 (data 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.

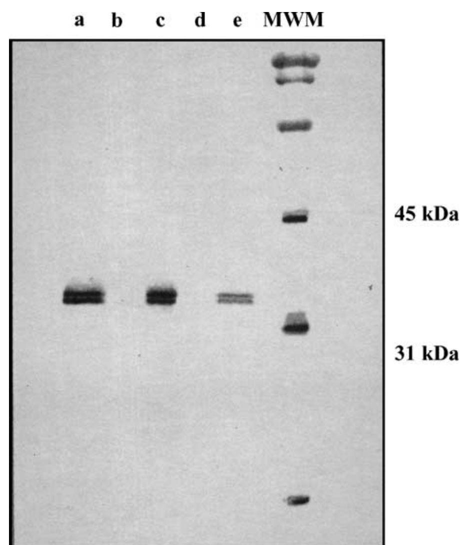

Fig. 3. Western blot analysis of HA/P expression in culture supernatants of SG25-1a, the *hapA* : : *celA* derivatives and their transformants with pCH2. Lane a, strain SG25-1a; lane b, strain L911; lane c, strain L911 transformed with pCH_2; lane d, strain L912; lane e, strain L912 transformed with pCH_2.

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.

Table 2
Extracellular protease activity of *V. cholerae* strains and their *hapA* : : *celA* derivatives

Strain[a]	Protease activity[b] ± S.D. (U)	Residual activity[c] (%)
SG25-1a	11.85 ± 0.07	100
L911	2.36 ± 0.12	19.94
81	19.87 ± 3.67	100
638	2.78 ± 0.42	14.01

[a] The protease activities of SG25-1 and SG25-1a are equivalent, as well as the activities of L911, L912, L911T and L912T.

[b] One unit of protease activity corresponds to the quantity of enzyme producing a net increase of one in the optical density of the sample in 1 h of reaction.

[c] Residual activity is expressed as percent respect to the parent strain.

Table 3
Colonising capacities of wild type virulent SG25-1 and derived cholera vaccine prototypes in the suckling mouse cholera model

Strain	Average per mouse[a,b]
SG25-1	5.5×10^6 a
SG25-1a	9.8×10^6 b
L911	1.9×10^5 b
L912	2.0×10^5 b
L911T	1.2×10^5 b
L912T	2.2×10^5 a

[a] Average of CFU recovered from the small intestine of mice. At least five mice were included in the analysis after being inoculated with 10^6–10^7 vibrios.

[b] Student *t*-test analysis: different letters (a and b) indicate statistically significant differences and the same letter means there are not such differences ($P < 0.05$).

3.3. 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).

3.5. Immunological analysis

Results are presented in Table 4. Antibody titres of rabbit antisera against O139 LPS were determined in an IgG-ELISA, and their vibriocidal activities against a homologous strain were assessed. As expected, the majority of infected rabbits seroconverted. At day 14, high geometric mean titres (GMT) of anti LPS IgG were detected, with the peak observed at day 28. Post infection titres of strain L911 were statistically lower than for the rest, but vibriocidal antibodies generated by all evaluated strains showed no statistically significant differences (Table 4). L911, L912, L911T and L912T elicited vibriocidal antibodies with similar magnitude titres, which showed a four-fold increase by day 7, reached a peak at day 14 in all but one instance of strain L912T (when the highest titter was evident by day 21) and remained high up to day 28.

4. Discussion

The CTXΦ prophage–RS1 arrays differ widely among pathogenic O1 and O139 by both, the number and relative

Table 4
Immune response to L911, L912, L911T and L912T in rabbits intraduodenally inoculated with a single dose of 10^9 CFU of each vaccine candidate[a]

Strain	Vibriocidal antibodies[b]		Anti-LPS IgG[b]	
	Day 14	Day 28	Day 14	Day 28
L911	127 (80–160)	160 (160)	174 (100–400)	224 (100–400)
L912	177 (80–640)	216 (40–160)	400 (100–800)	1008 (800–3200)
L911T	320 (80–1280)	127 (20–640)	2700 (1600–3200)	9050 (6400–12800)
L912T	80 (40–160)	127 (80–320)	400 (400)	3200 (3200–12800)

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

[a] Each value represents the average of at least three rabbits.

[b] GMT, geometric mean titre.

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 mechanism(s) 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-titters 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 *rstR* 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 superfluos 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 since the technique for identification is rapid, simple and sensitive. Loss of *celA* tags has not been observed after extensive passage in vitro and in vivo [11]. In prior experiments Robert et al. [11] concluded that inactivation of *hapA* had a positive effect on bacterial colonisation but our experiments with L911 and L912 did not reproduce such results what suggests that the effect of this mutation in the enhancement of bacterial colonisation is dependent on the strain analysed.

V. cholerae SG25-1 colonises the small bowel of the infant mouse cholera model but at lower counts than O1 *V. cholerae* in similar experiments as we previously reported [11]. Although *hapA* mutants colonise the infant mice intestines, they showed a 10-fold decrease in their colonising abilities. However, they appeared to interact with the intestinal immune system of animal models and elicit serological responses comparable to those seen with their parental strains (Table 4).

The geometric mean titres of anti LPS IgG antibodies elicited by L911 are lower than the elicited by the rest of the vaccine candidates. In contrast, no differences were detected in the vibriocidal activity. Previous studies in human volunteers [46] showed that primary vibriocidal responses seemed to correlate better with IgM titres than with IgG titres. This suggests to be the case for L911 and indicates a possible weaker interaction of this filamentous strain with the intestinal immune system.

Other authors indicated that O139 strains were encapsulated and consequently more resistant to complement mediated bacteriolysis. Their analysis provided an explanation for the relatively low levels of vibriocidal antibodies found against target strain SG25-1 in vibriocidal tests. Additional experiments [47] showed that capsular material might not be the only responsible for the apparent resistance of O139 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 reactogenic 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 = reactogenicity" 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 reactogenic factor. Wu et al. have shown that HA/P can act as a cytotoxin that perturbs the barrier function of epithelial cells (MDCK-1) 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-reactogenic 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 reactogenicity. We consider that the overall pathogenesis of *V. cholerae* is a complex issue and more investigation is needed to understand the exact role of HA/P 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 O395, exhibited a vibriocidal response of very low titre and no anti-toxin seroconvertion 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 pili (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*. 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 CTXΦ phage results 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 CTXΦ deletions, as a suitable combination to produce a protective O139 vaccine, with enough safety for human use and environmental release.

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Novel Type of Specialized Transduction for CTXϕ or Its Satellite Phage RS1 Mediated by Filamentous Phage VGJϕ in *Vibrio cholerae*

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The main virulence factor of *Vibrio cholerae*, the cholera toxin, is encoded by the *ctxAB* operon, which is contained in the genome of the lysogenic filamentous phage CTXϕ. This phage transmits *ctxAB* genes between *V. cholerae* bacterial populations that express toxin-coregulated pilus (TCP), the CTXϕ receptor. In investigating new forms of *ctxAB* transmission, we found that *V. cholerae* filamentous phage VGJϕ, which uses the mannose-sensitive hemagglutinin (MSHA) pilus as a receptor, transmits CTXϕ or its satellite phage RS1 by an efficient and highly specific TCP-independent mechanism. This is a novel type of specialized transduction consisting in the site-specific cointegration of VGJϕ and CTXϕ (or RS1) replicative forms to produce a single hybrid molecule, which generates a single-stranded DNA hybrid genome that is packaged into hybrid viral particles designated HybPϕ (for the VGJϕ/CTXϕ hybrid) and HybRSϕ (for the VGJϕ/RS1 hybrid). The hybrid phages replicate by using the VGJϕ replicating functions and use the VGJϕ capsid, retaining the ability to infect via MSHA. The hybrid phages infect most tested strains more efficiently than CTXϕ, even under in vitro optimal conditions for TCP expression. Infection and lysogenization with HybPϕ revert the *V. cholerae* live attenuated vaccine strain 1333 to virulence. Our results reinforce that TCP is not indispensable for the acquisition of CTXϕ. Thus, we discuss an alternative to the current accepted evolutionary model for the emergence of new toxigenic strains of *V. cholerae* and the importance of our findings for the development of an environmentally safer live attenuated cholera vaccine.

The filamentous phage CTXϕ contains the *ctxAB* genes encoding cholera toxin (CT), the main virulence factor of the pathogenic gram-negative bacterium *Vibrio cholerae* (49). In toxigenic El Tor and O139 strains of *V. cholerae* CTXϕ is integrated at the *dif* site in the bacterial genome arrayed in different tandem structures along with the related satellite phage RS1 (11, 39). The genome of RS1 is a short version of the genome of CTXϕ, which contains genes encoding proteins needed for replication (RstA), integration (RstB), and regulation of gene expression (RstR and RstC) but lacks the genes encoding proteins needed for assembling and secretion of viral particles (Psh, Cep, $pIII^{CTX}$, Ace, and Zot), as well as CT, which is not necessary for phage morphogenesis (11). Thus, satellite phage RS1 can replicate autonomously but depends on its helper phage CTXϕ for assembly and secretion and thereby for transmission of RS1 viral particles (11). Conversely, RS1 encodes the antirepressor RstC, which is not present in CTXϕ (9). This protein promotes transcription of CTXϕ and RS1 genes by counteracting the activity of the phage repressor RstR (9). Thus, RS1 enhances transmission of both CTXϕ and itself by means of RstC antirepressor activity (9).

Classical strains of *V. cholerae* contain nonfunctional CTXϕ prophages, whereas El Tor and O139 strains contain fully active prophages that produce infective CTXϕ viral particles (10). CTXϕ site specifically integrates into the host chromosome by a process dependent on the host recombinases XerC and XerD, which ordinarily catalyze the resolution of chromosome dimers at the *dif* recombination site (25). Other filamentous phages such as f237 of *Vibrio parahaemolyticus*, ϕLf of *Xanthomonas campestris*, Xfϕf1 of *Xilella fastidiosa*, CUSϕ-2 of *Yersinia pestis*, and VGJϕ of *V. cholerae* seem to exploit the XerCD recombination system to integrate into the chromosome of their hosts, suggesting that lysogenic filamentous phages are more common than initially thought (5, 25, 26).

CTXϕ infects *V. cholerae* through toxin-coregulated pilus (TCP) (49), a type IV pilus essential for intestinal colonization (47) that is encoded by a gene cluster contained in the *V. cholerae* pathogenicity island (VPI) (33). Although VPI seems to move horizontally between bacterial populations of *V. cholerae*, the transfer mechanism is still controversial. Karaolis et al. presented data suggesting that VPI is the prophage state of another filamentous phage, VPIϕ, which they thought to use TcpA as major capsid protein (34); however, this hypothesis has raised several unanswered questions (11), and the results from that study could not be reproduced by other researchers (16). Perhaps VPI transmission is mediated by several mechanisms that follow different pathways; for example, O'Shea and Boyd have found that VPI can be mobilized by the generalized transducing phage CP-T1 (43); however, the main mechanism accounting for VPI transmission probably has not been discovered yet. Whatever the mechanism, VPI, carrying the CTXϕ receptor, also has the ability to move horizontally between bacterial populations, providing CTXϕ with the advantage of

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