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Tagging a *Vibrio cholerae* El Tor candidate vaccine strain by disruption of its hemagglutinin/protease gene using a novel reporter enzyme: *Clostridium thermocellum* endoglucanase A

Alma Robert*, Anisia Silva†, Jorge A. Benitez*¶, Boris L. Rodriguez*, Rafael Fando*, Javier Campos*, Dilip K. Sengupta‡§, Mary Boesman-Finkelstein‡ and Richard A. Finkelstein‡

The celA *gene encoding* Clostridium thermocellum *endoglucanase A was expressed in* Vibrio cholerae *on its own promoter and used to tag a candidate El Tor biotype cholera vaccine strain. Colonies of the tagged strain could be unequivocally distinguished by overlaying them with CM-cellulose indicator agar and Congo Red staining. Expression of* celA *did not affect growth of* V. cholerae in vitro *and* in vivo. *The* celA *gene was inserted in the chromosomal* hap *locus encoding* V. cholerae *hemagglutinin/protease, a putative "detachase", to create a* hap$^-$ *mutant that could be identified and scored by its halo of cellulolytic activity. The inactivation of* hap *had a positive effect on colonization in the infant mice model. The above results indicate that* celA *is a suitable marker gene for* V. cholerae *and* hap *is an appropriate locus for insertion of foreign DNA in vaccine development. Inactivation of* hap, *by increasing the duration of adherence, might decrease excretion of the resulting vaccine vector strain and thus increase its immunogenicity.*

Keywords: *Vibrio cholerae*; vaccine; hemagglutinin/protease; endoglucanase A; reporter gene; *in vivo* marker exchange

Vibrio cholerae of serogroups O1 and O139, the causative agents of cholera, secrete a potent enterotoxin that causes the life-threatening diarrhea of cholera[1]. Cholera toxin and other putative toxic factors are clustered in the bacterial chromosome and flanked by repeat sequences forming a virulence cassette[2]. The recent spread of the seventh pandemic, characterized by the predominance of El Tor vibrios, to the western hemisphere and the emergence of serogroup O139 in India, re-emphasize the need for a safe and effective vaccine. A current strategy to develop cholera vaccines is to delete attributes of virulence to create attenuated strains[3]. Resultant candidate vaccine strains have elicited significant antibacterial protection against experimental cholera challenge[3]. Unfortunately, most El Tor candidate vaccine strains, even those lacking the whole virulence cassette, have been considered to be too reactogenic for wide scale usage[4].

Recently, several genetically engineered O1 El Tor and O139 candidate vaccine strains have been claimed to be well tolerated and immunogenic in volunteer studies[5,6]. The efficacy of these experimental vaccines remains to be confirmed by field trials in endemic areas.

In order to facilitate field experiments it is desirable to mark vaccine strains with a reporter gene that could allow their rapid and unequivocal distinction from other cholera vibrios present in the environment. A suitable reporter gene would also facilitate the study of complex phenotypic traits such as virulence and colonization capacity. An appropriate reporter gene should encode an activity not present in other enteric bacteria, not toxic for *in vitro* or *in vivo* growth of the bacterium and easy to detect.

The *celA* gene from *Clostridium thermocellum* encodes a thermophilic $\beta(1\rightarrow4)$ glucanglucanohydrolase denoted endoglucanase A[7]. The activity of this enzyme can be readily assayed in crude extracts, polyacrylamide gels and colonies grown on solid media[7]. We have previously

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Table 1 Bacterial strains and plasmids

Strain	Description and remarks	Reference
E. coli:		
MC1061	hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi, host for general purpose cloning vectors.	16
SY327λpir	Δ(lac pro) argE(Am) rif nalA recA56 (λpirR6K), host for suicide vectors pGP704 and pGPH6.	17
SM10λpir	thi thr leu tonA lacY supE recA::RP4-2Tc::Mu (λpirR6K) Km^r, host for suicide vectors with transfer functions integrated in chromosome.	18
V. cholerae:		
81	Δ(ctxA ctxB zot ace orfU cep) mutant from El Tor Ogawa Perú strain C7258.	19
815	Trimethoprim^r, thymidine-requiring mutant derived from strain 81.	This study
63	Amp^r co-integrate obtained by integration of pGPH6 to the *hap* locus of strain 81.	This study
638	Amp^s segregant isolated from strain 63 containing the insertionally inactivated hap gene (*hap::celA*).	This study
Plasmids:		
pCT104	Amp^r, 3.2 kb *Cl. thermocellum* HindIII fragment encoding endoglucanase A (*celA*) in HindIII site of pBR322.	7
pCH2	Cml^r, 3.2 kb *V. cholerae* 3083 fragment encoding hemagglutinin/protease cloned in pACYCC184.	14
pAH3	Cml^r, insertionally inactivated hap gene (*hap::celA*).	This study
pIJ2925	Amp^r, general purpose cloning vector for white–blue selection with expanded polylinker.	20
pIJHC1	Amp^r, insertionally inactivated *hap* flanked by BglII restriction sites.	This study
pGP704	Amp^r, suicide vector containing oriR6K and mobRP4 (mobilization) genes.	21
pGPH6	Amp^r, BglII fragment with insertionally inactivated *hap* in pGP704.	This study

reported the use of *Cl. thermocellum* endoglucanase A as a reporter enzyme to study gene expression and protein secretion in yeast[8,9].

V. cholerae produce a soluble hemagglutinin/protease (Hap)[10], a member of a family of zinc-dependent metalloproteases[11], that proteolytically activates cholera toxin A subunit[12], and hydrolyzes several physiologically important proteins such as mucin, fibronectin and lactoferrin[13]. The *hap* gene encoding this factor has been cloned and a *hap*$^-$ mutant retained full virulence in the infant rabbit model[14]. The lack of a functional *hap* gene increased the duration of adherence of cholera vibrios to cultured human intestinal cells[15]. Pretreatment of cultured intestinal cells with Hap prevented the attachment of vibrios[15]. Consequently, Hap could play a role in detachment and subsequent spread of the infecting vibrios[15].

In the present work we describe the stable integration of *celA* in the *V. cholerae hap* locus to create a *hap*$^-$ mutant that expressed an enzymatically active cell-associated *celA* protein. Cholera vibrios expressing *celA* appeared as red colonies surrounded by a transparent halo in a red field when overlayed with CM-cellulose indicator agar and stained with Congo Red. The *hap*$^-$ mutant created by insertion of *celA* showed increased colonization capacity in the infant mouse cholera model.

MATERIALS AND METHODS

Strains and media

Bacterial strains and plasmids are listed and described in *Table 1*. All strains were grown in LB medium and conserved at −70°C in tryptic-soy broth containing 25% glycerol. Ampicillin (100 μg ml^{-1}), polymyxin B (100 U ml^{-1}), chloramphenicol (34 μg ml^{-1}) or thymidine (50 μg ml^{-1}) were added when necessary.

Plasmid constructions and genetic techniques

The construction of suicide vector pGPH6 is shown in *Figure 1*. Plasmids pGP704 and pGPH6 were propagated in *Escherichia coli* SY327λpir or SM10λpir for conjugative transfer to *V. cholerae*. Other vectors were propagated in MC1061. *E. coli* was transformed as described by Sambrook *et al.*[22] Plasmid DNA was prepared according to Birnboim and Doly[23]. A 3.2 kb klenow-blunted HindIII fragment containing the *celA* gene was extracted from plasmid pCT104 and inserted in the StuI site of plasmid pCH2, located within the *hap* coding sequence, to create plasmid pAHC3. A 6.4 kb HindIII fragment encoding *hap::celA* was transferred to plasmid pIJ2925 to create pIJHC1, subsequently re-extracted as a BglII restriction fragment and inserted in the unique BglII site of suicide vector pGP704 to produce pGPH6. SM10λpir containing pGPH6 was filter-mated with strain 81 and *V. cholerae* exconjugants selected in LB plates supplemented with ampicillin and polymyxin B to select against donor *E. coli*. Chromosomal DNA from *V. cholerae* exconjugants was prepared as described by Ausubel *et al.*[24] *V. cholerae* pGPH6 exconjugants were characterized by Southern blot hybridization analysis using a DIG-DNA labeling and detection kit from Boehringer Mannheim. The exconjugant 63 was grown in LB broth without antibiotics, plated on LB agar, and ampicillin-sensitive segregant 638 isolated by replica-plating to LB agar supplemented with ampicillin. Strain 815 was isolated as a spontaneous mutant resistant to 200 μg ml^{-1} trimethoprim in LB agar as described by Miller[25]. DNA restriction and modification enzymes were from Boehringer Mannheim and used according to manufacturer's instructions.

Protein and enzymatic techniques

A suspension consisting of 1 g *V. cholerae* cells (wet wt), 1 mg of *p*-methylsulphonylfluoride, 2.7 ml of phosphate-citrate buffer pH 6.3 and 2.7 g of 0.10–0.11 mm glass beads was disrupted in a vortex mixer. The cell extract was cleared by high speed centrifugation and samples containing 100 μg of protein fractionated by SDS–PAGE according to Laemmli[26]. Endoglucanase A protein bands in western blots were visualized using a polyclonal antiserum raised against purified endoglucanase A from *Cl. thermocellum* and peroxidase-conjugated anti-rabbit IgG (whole molecule, Sigma Chemical Co.) Bands of endoglucanase A activity in CMC-agar replicas of polyacrylamide gels were detected

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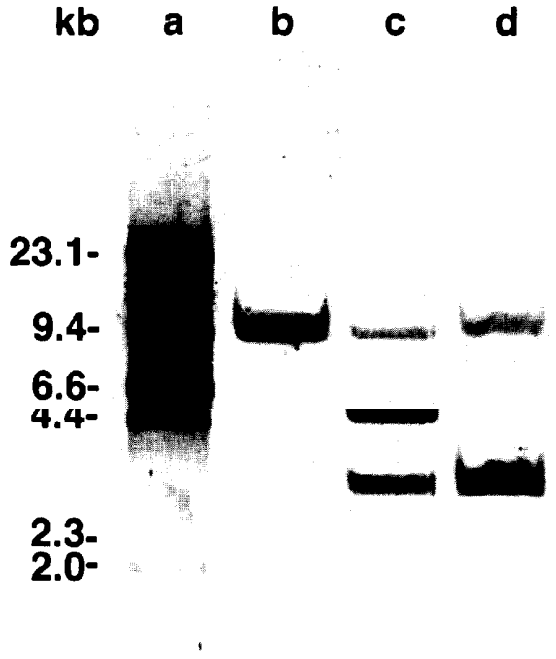

Figure 3 Southern blot hybridization analysis of *hap*⁻ mutants created by *celA* insertion. Lane a, molecular weight marker (λDNA digested HindIII); lane b, strain 81 DNA digested with BamHI; lane c, exconjugant 63 DNA digested with BamHI; lane d, DNA from segregant 638 digested with BamHI. The nitrocellulose filter was probed with a 3.2 kb HindIII fragment from plasmid pCH2 containing the *hap* gene

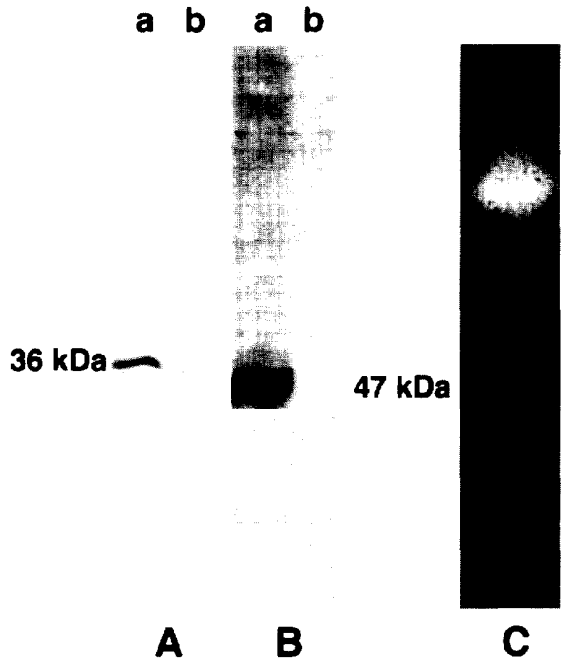

Figure 4 (A) Western blot analysis of Hap expression in *hap*⁻ mutants. Lane a, supernatant from strain 81; lane b, strain 638. (B) Western blot analysis of endoglucanase A expressed in *V. cholerae*. Lane a, crude extract from strain 638; lane b, crude extract from strain 81. (C) Detection of endoglucanase A activity in CM-cellulose agarose replicate of polyacrylamide gels of crude extracts of strain 638. In this case the sample was treated for 45 min at 60°C in loading buffer instead of boiling and the image was enhanced for additional contrast

BamHI site) within the ≈9.4 kb DNA fragment that contains *hap* in strain 81 (lane b). Both bands of the 638 BamHI digest hybridized with a *celA* probe (data not shown). The resulting insertion mutant lacked soluble hemagglutinating activity and the Hap band in western blots with specific anti-Hap serum (*Figure 4*A), and showed a marked decrease in proteolytic activity in gelatin and milk indicator plates (data not shown).

Expression of *celA* in *v. cholerae*

SDS–PAGE and western blot analysis of cell extracts from strain 638 revealed the presence of a 47 kDa immunoreactive protein (*Figure 4*B, lane a) not present in strain 81 (*Figure 4*B, lane b). The CM-cellulose-containing agarose replica of a nondenatured sample fractionated by SDS–PAGE (*Figure 4*C) indicated that, as observed in other systems[27], endoglucanase A forms enzymatically active high molecular weight aggregates. All the endoglucanase activity expressed in strain 638 was cell-associated: no activity being found in the supernatant (data not shown). *V. cholerae* colonies expressing endoglucanase A could be detected on LB agar plates after overlaying with CM-cellulose indicator agar, incubation at 60°C and staining with Congo Red. *celA*⁺ vibrios appeared as red colonies surrounded by a clear halo of cellulolytic activity in a red field (*Figure 5*).

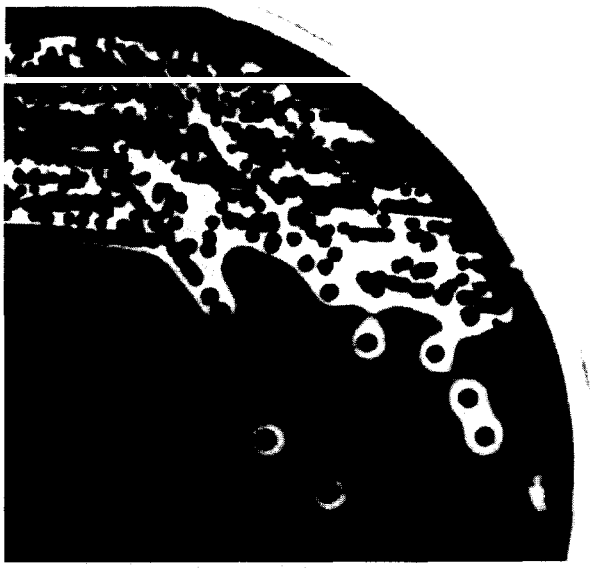

Figure 5 Detection of endoglucanase A in colonies of *celA*⁺ vibrios

Intestinal colonization of *hap*⁻ mutants generated by insertion of *celA*

Competitive colonization assays indicated that strain 638 lacking Hap colonized better in the infant mice than its parental strain 81 (*Table 2*). Strain 815, used as a negative control in these experiments, showed a nearly

Table 2 Competitive intestinal colonization assay for *V. cholerae* hemagglutinin/protease negative mutants created by insertion of *celA*

Strains inoculated	Intra-intestinal growth (c.f.u./intestine)[a]	Competitive index *in vivo*[b]	*in vitro*
81	7.0×10^{7}	—	—
638	3.8×10^{8}	—	—
815	1.4×10^{7}	—	—
638+81	—	1.53±0.28	1.10
815+81	—	0.13±0.03	1.06[b]

[a]The intestine homogenates of three independent mice were plated in triplicate. [b]Since 815 does not grow in the absence of thymidine, this index is the ratio of growth of mutant to wild type under nonselective conditions

tenfold decrease in colonization capacity under the same experimental conditions (*Table 2*). Intestinal growth of each of the strains separately in the descending order 63881815 (*Table 2*) was in good agreement with the competitive indexes. No effect on growth was observed under *in vitro* conditions. The specific growth rate for strains 81, 638, and 815 in LB broth (or LB plus thymidine for 815) were 0.30, 0.28, and 0.29 h^{-1}, respectively.

DISCUSSION

An enzymatically active thermophilic endoglucanase encoded by *Cl. thermocellum celA* gene was expressed in *V. cholerae* under the control of its own promoter. As expected from the allelic exchange scheme shown in *Figure 2*, disappearance of the Hap band in western blots (*Figure 4*A) was paralleled by the appearance of a protein reactive to anti-endoglucanase A serum (*Figure 4*B). The molecular weight of the *celA* protein synthesized in strain 638 was in close agreement with the size predicted from the DNA sequence for mature endoglucanase A. Thus, it is likely that endoglucanase A is processed in *V. cholerae* by removal of its signal peptide.

V. cholerae strains expressing *celA* can be unequivocally distinguished from other vibrios by overlaying colonies from CM-cellulose indicator agar (*Figure 5*). An advantage of using *celA* over dose-dependent resistance markers is that there is no background enzyme activity in unmarked vibrios. This rapid, simple and sensitive technique can detect less than ng levels of endoglucanase protein[27]. The above technique is particularly applicable in minimally equipped laboratories or research stations created for field experiments. Loss of the celA marker was not observed after extensive passage *in vitro* and *in vivo* (mice).

Growth defects due to cellular misfunction or metabolic stress can affect the colonization capacity and immunogenicity of live vaccine strains and have detrimental effects on vaccine efficacy[3]. Expression of the *celA* gene was not toxic for growth of *V. cholerae in vitro* when carried on a multicopy plasmid, integrated in exconjugant 63 (data not shown) or exchanged with the wild type *hap* locus in strain 638 (*Table 1*). Therefore, we suggest that *celA* is a suitable marker gene for *V. cholerae* live vaccine strains.

We constructed a *hap*$^-$ mutant by insertion of *celA*. The structure of the intermediate clone 63 and the final mutant was confirmed by Southern blot hybridization analysis (*Figure 3*). As mentioned, the insertion mutation did not affect growth of the bacterium under our defined conditions *in vitro* (*Table 2*). Previous analysis of *hap*$^-$ mutants based on lethality measurement in infant rabbits suggested that Hap is not a major virulence factor in this model[14]. A *hap*$^-$ mutant adhered for a longer period to cultured human intestinal cells than its parental *hap*$^+$ vibrio suggesting that Hap might have a 'detachase' function[15]. In order to test if this activity is expressed *in vivo* we used a different approach. The *hap* gene from the attenuated strain 81[19] was disrupted by *celA* insertion to create 638 and its capacity to grow in the infant mouse, instead of lethality, assessed. Since colonization depends upon intestinal adherence, a detachase$^-$ mutant would be expected to colonize better than its *hap*$^+$ parent. As shown in *Table 2*, inactivation of *hap* by *celA* insertion had a positive affect on bacterial colonization which, in this experimental model, is measured as the sum of both intraluminal and adherent bacteria in the homogenate (whereas in humans it would represent only the vibrios in the lumen, i.e. the stool). This result is in agreement with the hypothesis that *hap* encodes a 'detachase' activity that is expressed *in vivo*. *V. cholerae* detaches from the intestinal brush border in animal models[29,30]. If *hap* is responsible for this activity, one would expect a *hap*$^+$ phenotype to contribute to the epidemic potential of *V. cholerae* strains.

The present results suggest that *hap* is a recommendable locus for inserting foreign DNA in *V. cholerae* for antigen delivery to mucosal sites. *Hap* inactivation might enhance the duration of adherence and immunogenicity of the resulting vaccine vector strain. It is also conceivable that a *hap*$^-$ vaccine would diminish the spread of the vaccine strain and decrease its potential environmental impact. Interestingly, the classical biotype cholera vaccine strain CVD103HgR, derived from strain 569B that does not express Hap, is minimally excreted by volunteers[3]. Is it possible that its immunogenicity, which although not equivalent to wild type but is far better than one might expect from the low stool counts, is due to its persistent adherence to M cells in the gut? The use of *celA* to disrupt *hap* facilitated counting of mutant vibrios after growth in the infant mouse intestine and can be applied to study the role of other putative virulence and colonization factors. Because there is no totally predictive experimental animal model, such genetically engineered candidate vaccine strains will be evaluated for reactogenicity and immunogenicity in human volunteers (Benitez *et al.*, in progress).

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