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Construction and Analysis of a *Vibrio cholerae* δ-Aminolevulinic Acid Auxotroph Which Confers Protective Immunity in a Rabbit Model†

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***Vibrio cholerae* CVD101 is a very effective live vaccine. Although this strain does not produce active cholera toxin because of a mutation in the gene for the cholera toxin A subunit, it still shows residual pathogenicity. To attenuate CVD101 further, we set out to isolate derivatives of CVD101 which were limited in their ability to proliferate in vivo. Two δ-aminolevulinic acid auxotrophs of CVD101, designated V286 and V287, were isolated by transposon mutagenesis and penicillin enrichment. Southern blotting revealed that the mutants differed with respect to the location of the transposon insertion. Under aerobic conditions, in the absence of δ-aminolevulinic acid, both mutants showed diminished growth compared with CVD101. The growth of V286 was most severely affected. Microaerophilic growth of both mutants was less affected. Competition experiments with a rabbit model showed that strain V286 was found in numbers 10^3- to 10^4-fold lower than its parental strain. This observation indicates that strain V286 is impaired in its ability to colonize the rabbit intestine. It also supports an important role for aerobic growth in the colonization of the intestine by *V. cholerae*. Vaccination of rabbits with a single dose of strain V286 resulted in full protection against challenge with a virulent strain. Strain V286 was not shed from rabbits in a cultivatable form. Our results suggest that δ-aminolevulinic acid auxotrophy can attenuate *V. cholerae* by limiting its ability to colonize without affecting its capacity to induce protective immunity. Furthermore, this type of mutation may prevent the spread of *V. cholerae* vaccine strains in the environment.**

The presently used cholera vaccine consists of killed *Vibrio cholerae* cells and confers only moderate protection of a brief duration (11). Therefore, efforts are being made to develop an improved vaccine. Two experimental cholera vaccines, both of which have to be administered orally, are in development: a live vaccine based on attenuated, cholera toxin-defective *V. cholerae* strains (14, 15) and a whole-cell vaccine comprising killed *V. cholerae* cells and the B subunit of cholera toxin (3). Both vaccines have been tested in animal models and human volunteers. Field trials revealed that the whole-cell vaccine has restricted efficacy in children younger than 5 years (3). Furthermore, in the rabbit model that we use to study infection with and immunity to *V. cholerae*, the killed vaccine does not induce a protective immune response (8, 9). A live vaccine composed of a *V. cholerae* strain designated CVD101, in which the gene for the A subunit of cholera toxin has been inactivated (i.e., a *ctxA* mutant strain), was able to induce protective immunity in human volunteers (14), and this vaccine was also found to be protective in our animal model (8). Thus, CVD101 is a very promising vaccine candidate. However, this strain still caused diarrhea in half of the recipients (14). This residual pathogenicity may be due to the production of toxins, distinct from cholera toxin, such as Shiga-like toxin (15). CVD103, a *ctxA* mutant strain which does not produce Shiga-like toxin, caused fewer side effects and conferred full protection in human volunteers (15). A derivative of CVD103 (CVD103-HgR) which contains a mercury resistance gene in the hemolysin locus was able to induce a vibriocidal antibody response, and the occurrence of adverse effects in vaccinees was further reduced (4, 15, 17). However, in some field trials poor immunogenicity of CVD103-HgR has been noted (23).

These studies show that undesirable side effects of live vaccine strains may be reduced by inactivating toxin genes. Another, complementary, approach involves the inactivation of bacterial genes which are required for proliferation in the host (7). Because bacteria carrying such mutations are limited in their capacity to colonize, exposure of the host to toxins produced by the pathogen will also be reduced. Such mutants may be especially useful when side effects are not due to one or more distinct factors but are inherent in the intensive colonization of the small intestine. Finally, mutations that limit growth in the host may also prevent live vaccine strains from disseminating in the environment. Immunization with *V. cholerae ctxA* mutant strains limited for in vivo growth because of thymidine auxotrophy or recombination deficiency did not cause any side effects in human volunteers (13, 14). However, the immunogenicity of these strains was impaired, presumably because they were unable to persist long enough in the host. The aim of this study was to construct strains which were less restricted in their ability to proliferate in vivo. We anticipated that mutations affecting the synthesis of cofactors of enzymes might allow the bacteria to grow for a number of generations under nonpermissive conditions (i.e., to coast). We presumed that growth would continue until the active enzymes (i.e., enzymes with

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† This article is dedicated to Piet Guinée, deceased, who initiated this work and whose enthusiasm and creativity played such an important role in its progress.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
Strains		
V. cholerae		
C5	Ogawa, El Tor	9
CVD101	*ctxA*; Ogawa, classical	12
V286	*xxx*::Tn*286*; ALA auxotroph; Kan^r	This study
V287	*yyy*::Tn*287*; ALA auxotroph; Kan^r	This study
E. coli		
MM294	*endA hsdR pro supF*	21
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu	19
Plasmids		
pRT291	IncP1 Tn*phoA* Tet^r Kan^r	21
pPH1JI	*tra* IncP1 Spe^r Gen^r Str^r	1

cofactors) were diluted by cell division to a level incompatible with growth. Heme mutants seemed promising in this respect, because heme is a cofactor for many enzymes, such as cytochromes and catalases.

δ-Aminolevulinic acid (ALA) is an important intermediate in the biosynthesis of heme, and ALA auxotrophs of *Escherichia coli* and *Salmonella typhimurium* have been shown to be impaired in the expression of heme-requiring enzymes (6). Therefore, we decided to isolate *V. cholerae* strains which require exogenous ALA for growth. Here we describe the isolation of *V. cholerae* ALA auxotrophs and their characterization in vitro and in vivo. We show that a *V. cholerae* ALA auxotroph, although affected in its ability to persist in the host, conferred protective immunity in the rabbit model after vaccination with a single dose.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *V. cholerae* strains were cultured on 5% sheep blood agar or Monsur agar (18). Monsur agar was prepared by sterilization (15 min at 120°C) of a solution composed of 10 g of Casitone (Difco) per liter, 10 g of NaCl per liter, 5 g of sodium taurocholate per liter, 1 g of $NaHCO_3$ per liter, 30 g of gelatin per liter, and 18 g of agar per liter. Subsequently, the solution was cooled to 50°C, and 0.5 ml of a 1% potassium tellurate solution per liter was added. The potassium tellurate solution had been filter sterilized. Liquid culturing was done with nutrient broth (Difco) and minimal medium (MM; 15 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 6 g of KH_2PO_4 per liter, 1 g of Na_2SO_4 per liter, 2 g of NH_4Cl per liter, 5 g of glucose per liter, 0.1% solubilized trace salts, 1% amino acids for basal Eagle medium [ICN Flow, Irvine, United Kingdom]). The trace salts solution contained 10 g of $MgSO_4 \cdot 7H_2O$ per liter, 1 g of $MnCl_2 \cdot 4H_2O$ per liter, 1 g of $FeCl_3 \cdot 6H_2O$ per liter, and 0.40 g of $CaCl_2 \cdot 2H_2O$ per liter. Antibiotics were used in the following concentrations: kanamycin, 50 μg/ml; gentamicin, 30 μg/ml; penicillin, 300 μg/ml; and tetracycline, 25 μg/ml. ALA (Janssen Pharmaceutica, Beerse, Belgium) was added to a final concentration of 50 μg/ml. *V. cholerae* strains were cultured at 25°C when they were used to infect rabbits, because we have observed that the minimal infective dose at this temperature is approximately 10,000-fold lower than that at 37°C (9). For all other experiments, strains were grown at 37°C.

For liquid *V. cholerae* cultures, a colony from a blood agar plate was washed twice with phosphate-buffered saline (PBS; 1.44 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 0.27 g of KH_2PO_4 per liter, 8.7 g of NaCl per liter [pH 7.4]), suspended in 1 ml of PBS, and used to inoculate the appropriate media. Strain CVD101 was cultured in MM, whereas ALA auxotrophs were cultured in either MM supplemented with kanamycin (MMk) or MM supplemented with ALA and kanamycin (MMak). To ensure proper aeration, we filled 500-ml flasks with 50 ml of medium and incubated them in a gyratory shaker (200 rpm, 37°C). A microaerophilic environment was obtained by filling 100-ml flasks with 100 ml of medium. To study the ability of the ALA auxotrophs to continue growth in the absence of ALA (i.e., to coast), we grew strains in MMak, harvested them, and washed them three times with PBS. Subsequently, MMk was inoculated to an optical density at 600 nm (OD_{600}) of 0.02 (approximately 2×10^7 cells per ml). A similar procedure was used to deplete cells of heme.

Isolation of *V. cholerae* ALA auxotrophs. ALA auxotrophs of *V. cholerae ctxA* mutant strain CVD101 were isolated by transposon mutagenesis with Tn*phoA* (16) and enriched by counterselection with penicillin. Transposon mutagenesis was performed as described by Taylor et al. (21). In brief, donor strain SM10 carrying pRT291 was mated with *V. cholerae* CVD101, and the mating mixture was streaked on Monsur agar supplemented with tetracycline. To select for the transposition of Tn*phoA* from pRT291 to the chromosome of *V. cholerae*, we mated CVD101 harboring pRT291 with MM294 carrying the gentamicin resistance plasmid pPH1JI, which is incompatible with pRT291. The mating mixture was plated on Monsur agar supplemented with ALA, gentamicin (to select for pPH1JI), and kanamycin (to select for Tn*phoA*). Gentamicin- and kanamycin-resistant CVD101 strains were harvested and suspended in a small volume of MM, and the suspension was incubated for 3 h at 37°C in a gyratory shaker to deplete ALA auxotrophs of endogenous heme. Subsequently, penicillin was added to the suspension and 10^{-2} and 10^{-4} dilutions of the suspension, and incubation was continued for 3 h at 37°C. This procedure eliminated *V. cholerae* cells which were still able to proliferate in the absence of ALA. Finally, after washing was done, various dilutions of bacteria were plated on Monsur agar containing kanamycin and ALA. After 72 h, colonies were replicated on Monsur agar with and without ALA. Colonies which grew only on plates with ALA were considered ALA auxotrophs.

DNA methods. Chromosomal DNA from *V. cholerae* was isolated as described by Wilson (22). For characterization of Tn*phoA* insertions, chromosomal DNA was digested with *Acc*I, separated electrophoretically overnight on an 0.8% agarose gel containing ethidium bromide (500 ng/ml), denaturated, and transferred to a Hybond N+ membrane (Amersham International plc, Amersham, United Kingdom) by vacuum blotting (Milliblot V-system; Millipore Corp., Bedford, Mass.). A 3.4-kbp *Hin*dIII fragment derived from Tn*5* was used as a probe (5). Digoxigenin labelling, hybridization, and detection of the probe were performed as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany). Hybridization was done overnight at 65°C with 1 ng of probe per ml. After two washing steps, the membrane was incubated with anti-digoxigenin antibodies conjugated to alkaline phosphatase (1:10,000). Binding of the probe was visualized by chemiluminescence

with 3-(2′-spiro-adamantane)-4-methoxy-(3″-phosphoryloxy) phenyl-1,2-dioxetane (Tropix Inc., Bedford, Mass.) as a substrate. Emitted light was detected with X-Omat film (Kodak Co., Rochester, N.Y.).

DNA sequences. Tn*5* and Tn*phoA* sequences were obtained from the EMBL data bank (accession numbers V00614 to V00618 and X04586, respectively).

Rabbit model. The surgical procedures used in the rabbit model were essentially those described by Spira et al. (20) for the RITARD (removable intestinal tie-adult rabbit diarrhea) model, with a few modifications (8, 9). In brief, after starvation for 24 h and anaesthesia, the cecum was ligated and *V. cholerae* cells were injected into the lumen of the duodenum about 10 cm distal to the stomach in a volume of 10 ml of PBS. The duodenal site of injection was found to be essential to obtain reproducible results. When rabbits were vaccinated, the ligation of the cecum was omitted.

Competitive growth in vivo. The effect of ALA auxotrophy on the ability of *V. cholerae* to colonize the rabbit intestine was studied by assessing competition between strain CVD101 and the ALA auxotroph. CVD101 and the ALA auxotroph were cultured overnight at 25°C in MM and MMak, respectively. Subsequently, cells were harvested and suspended in PBS. A mixture containing 10^5 or 10^6 cells of each strain in 10 ml of PBS was introduced by duodenal inoculation into the intestines of rabbits which had undergone cecal ligation. At 24, 48, 72, and 96 h after duodenal inoculation, two rabbits were sacrificed. The duodenum, jejunum, ileum, and colon were divided into segments of approximately 10 cm by ligation. Also, a segment was made of the cecal entrance, located between the ligated part of the cecum and the colon. PBS (5 ml) was injected into the segments through the intestinal wall by use of a hypodermic needle, and the segments were rubbed between thumb and forefinger to detach bacteria. The bacterial suspensions and dilutions of the suspensions were tested for the presence of the inoculated strain directly on Monsur agar or after enrichment. The ALA auxotroph was detected on Monsur agar containing kanamycin and ALA. Enrichment was performed by inoculation of nutrient broth supplemented with ALA and kanamycin for the isolation of the ALA auxotroph and incubation overnight at 37°C. Subsequently, dilutions were plated on Monsur agar as described above. Fecal pellets were tested in a similar manner. Slide agglutination was used for serotyping of colonies. Serum for slide agglutination was prepared by immunizing rabbits with heat-killed (60 min, 100°C) cells from strain C5. The serum was made specific for the Ogawa serotype by absorption with the Inawa strain.

Vaccine efficacy of ALA auxotroph V286. The ability of ALA auxotroph V286 to induce protective immunity against challenge with virulent *V. cholerae* was assessed in the rabbit model. For vaccination, animals were duodenally inoculated with different quantities of cells which had been cultured in MMak or MMk to deplete them of endogenous heme (see above). Rabbits were inoculated with 10^3 (MMk-cultured cells only), 10^5, or 10^7 cells. Five weeks after vaccination, the cecum was ligated and the rabbits were challenged by duodenal inoculation with 10^5 cells of virulent *V. cholerae* C5. This dose represents at least 100 50% lethal dose units (8). Rabbits were sacrificed 4 days after challenge or earlier if severe diarrhea was evident. At autopsy, the intestine was sampled and bacterial counts were determined for the intestinal contents to detect the challenge strain. Samples were taken from the duodenum, jejunum, ileum, colon, and cecum.

Statistical analysis. Statistical analysis was performed with Fisher's exact test by use of the program InStat (Graphpad, San Diego, Calif.).

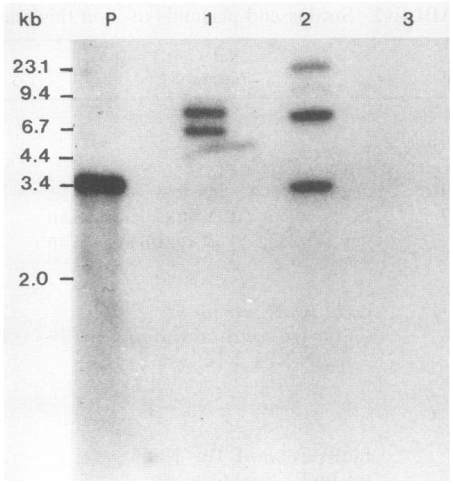

FIG. 1. Southern blot analysis of *V. cholerae* Tn*phoA* mutants. Chromosomal DNAs from CVD101, V286, and V287 were digested with *Acc*I and probed with a 3.4-kbp *Hin*dIII fragment from Tn*5*. Lanes: P, 3.4-kbp *Hin*dIII fragment from Tn*5*; 1, V286; 2, V287; 3, CVD101. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs. The largest band in lane 2 represents a partially digested fragment.

RESULTS

Isolation of ALA auxotrophs. After transposon mutagenesis of CVD101 and counterselection for ALA auxotrophs (see Materials and Methods), cells were plated on Monsur agar containing kanamycin and ALA. Subsequently, 400 colonies were replicated on Monsur agar plates with and without ALA, and 2 colonies which displayed growth in the presence of ALA only were found. These ALA auxotrophs were designated V286 and V287.

Detection of Tn*phoA* in ALA auxotrophs. Southern blotting was used to determine whether transposition to the chromosome of *V. cholerae* had occurred. A 3.4-kbp DNA fragment derived from Tn*5* was used as a probe. Chromosomal DNA was digested with *Acc*I, which should cleave Tn*phoA* in three fragments that react with the probe: a central fragment of 1.4 kbp and two flanking fragments containing Tn*phoA* and *V. cholerae* DNA. Unexpectedly, the central fragment was not detected in the two ALA auxotrophs (Fig. 1), suggesting that one of the two *Acc*I sites in Tn*phoA* has been lost through mutation or modification. Control experiments revealed that both *Acc*I sites were present in the Tn*phoA* copy carried by pRT291 (data not shown). Differently sized fragments hybridized to the Tn*5*-derived probe in strains V286 and V287, indicating that they contained Tn*phoA* at different locations on their chromosomes.

Growth characteristics of ALA auxotrophs in vitro. Aerobic growth of V286 and V287 in MM with and without ALA was compared with that of parental strain CVD101 (Fig. 2). In the absence of ALA, growth of V286 was not detected, while growth of V287 was reduced compared with that of CVD101. Apparently V287 has a leaky phenotype. The observed growth defects could be suppressed by the addition of ALA to the growth medium. The fact that growth of V286 in the absence of ALA was not detected may have been due to the low initial concentration of bacteria. Therefore, the experi-

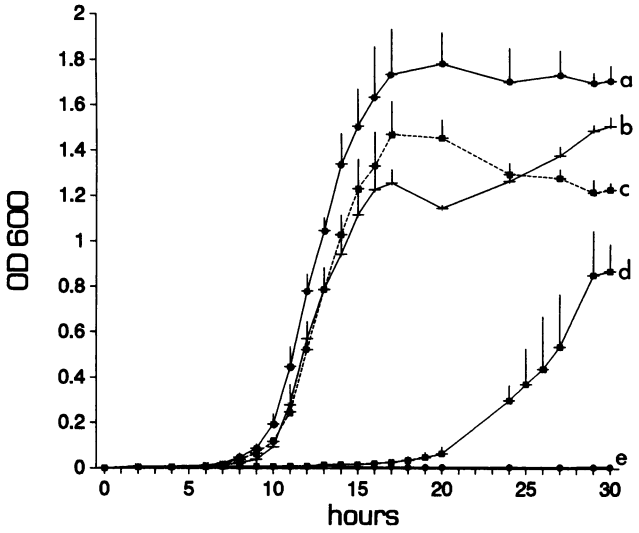

FIG. 2. Effect of ALA auxotrophy on growth in vitro. Cells were diluted in the appropriate medium to an OD_{600} of 0.001 (approximately 10^6 cells per ml) and grown aerobically at 37°C. ALA auxotrophs were cultured in the presence or absence of ALA. Each curve was based on three independent experiments. Standard deviations are indicated. (a) V286 with ALA. (b) CVD101. (c) V287 with ALA. (d) V287 without ALA. (e) V286 without ALA.

ment with V286 was repeated with an inoculum that was 20 times larger (Fig. 3). Under these conditions, a 20-fold increase in the OD_{600} was observed before growth stopped. This result suggests that V286 is able to continue growth (i.e., to coast) for approximately four generations in the absence of ALA. Most probably, this coasting is explained by the dilution of heme-containing compounds at cell division until their concentration becomes too low for further growth.

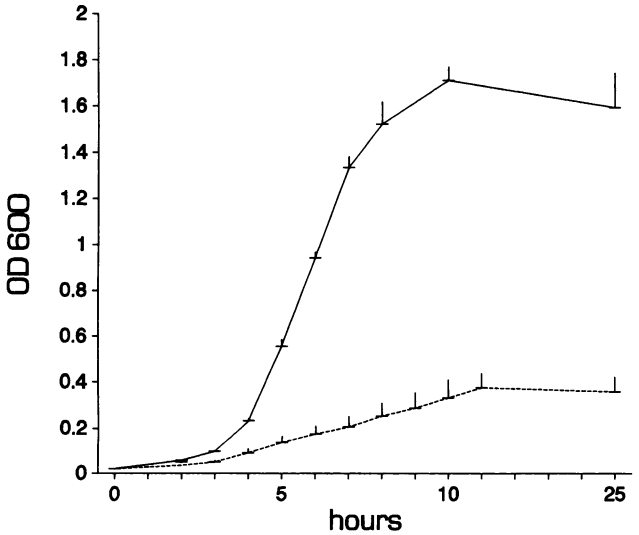

FIG. 3. Ability of strain V286 to coast. V286 was cultured in MMak, harvested, washed, and used to inoculate MMak (with ALA) (——) and MMk (without ALA) (- - - -) to an OD_{600} of 0.02 (approximately 2.10^7 cells per ml). Cells were grown aerobically at 37°C. Each curve was based on three independent experiments. Standard deviations are indicated.

Under microaerophilic conditions, in the absence of ALA, the differences in growth between CVD101 and both ALA auxotrophs were less pronounced (results not shown).

Competitive growth in vivo. Competition experiments were performed to determine the influence of ALA auxotrophy on the ability of *V. cholerae* to colonize the rabbit intestine. Since V287 had a leaky phenotype, it was not included in these experiments. CVD101 and V286 were cultured overnight at 25°C in MM and MMak, respectively. Cells were harvested and suspended in PBS, and a mixture of CVD101 and V286, containing 10^5 or 10^6 cells of each strain, was used for duodenal inoculation. At 24 to 96 h after duodenal inoculation, the numbers of viable CVD101 and V286 cells were determined in different parts of the intestinal tract and in the feces (Table 2). Similar results were obtained with both dilutions. At 24 h after duodenal inoculation, the parental strain had markedly outgrown V286 and was detected in numbers 3 or 4 orders of magnitude larger than the ALA auxotroph. Except in two instances, V286 could not be recovered from the intestinal tract after 48 h. In contrast, parental strain CVD101 remained present in the intestinal tract for up to 96 h. Furthermore, V286 cells could not be recovered from the feces even after enrichment. In contrast, CVD101 cells could be detected in the feces until day 2 of the experiment. Thus, compared with the parental strain, the ALA auxotroph was severely inhibited in its ability to persist in the rabbit intestine.

Protective effect of vaccination with V286. The protective effect of vaccination with V286 was studied in 12 rabbits. V286 was cultured in the presence and absence of ALA. The latter growth condition was used to minimize proliferation in vivo by depleting cells of endogenous heme. Five weeks after vaccination, rabbits were challenged with *V. cholerae* C5; four untreated rabbits served as controls (Table 3). The controls were not protected: three rabbits succumbed within 48 h, and one rabbit developed severe diarrhea. At autopsy, large numbers of *V. cholerae* were found in the intestinal tracts of all unvaccinated rabbits (Table 3). None of the 12 vaccinated rabbits showed disease symptoms and thus were fully protected. At autopsy, the challenge strain could only be detected after enrichment in two rabbits. Interestingly, immunization with V286 cells, which had been depleted of ALA, resulted in full protection. Apparently depletion of ALA does not impair the ability of V286 to elicit a protective immune response.

DISCUSSION

Studies with human volunteers have revealed great potential for live *V. cholerae ctxA* mutant strains as single-dose oral vaccines. However, these strains still cause moderate diarrhea in human volunteers, and we are attempting to attenuate them further by reducing their ability to proliferate in vivo. For the reasons explained above, we used a *ctxA* strain (CVD101) to isolate mutants auxotrophic for ALA. *E. coli* and *S. typhimurium* ALA auxotrophs are unable to grow aerobically under restrictive conditions. However, anaerobic growth of these mutants is unimpaired (6). It has been suggested that the effect on aerobic growth is due to oxidative damage, since ALA auxotrophs are deficient for catalase (6). The ability to grow anaerobically does not necessarily limit the usefulness of *V. cholerae* ALA auxotrophs as live vaccines, since aerobic growth may play an important role in the colonization of the intestine. Studies of the rat intestine present evidence for a high oxygen tension near the epithelial cell layer (2). Thus, it is conceivable that the

TABLE 2. In vivo competition between CVD101 and V286 in the rabbit intestine[a]

Strain detected	Time after inoculation (h)[b]	Dose[c]	Recovery[d] of CVD101 or V286 in:					
			Duodenum	Jejunum	Ileum	Cecum[e]	Colon	Feces
CVD101	24	10^5	2.9, +	4.6, 7.3	6.5, 7.0	4.7, 6.7	3.0, 6.2	+
		10^6	−, 5.7	6.2, 5.7	6.3, 6.7	+, +	4.0, 5.3	NT
	48	10^5	−, −	4.7, 6.0	6.0, 6.8	−, 4.7	−, +	+
		10^6	−, 3.0	5.2, −	8.0, 4.8	+, −	−, −	NT
	72	10^5	−, −	1.7, 7.4	5.7, 5.9	−, −	−, +	−
		10^6	−, 6.8	3.2, 6.3	5.6, 8.4	−, +	0.6, 6.0	NT
	96	10^5	−, −	−, −	3.4, 1.5	−, +	−, −	NT
		10^6	−, −	−, −	−, 6.0	−, +	−, 5.8	NT
V286	24	10^5	−, −	1.7, 3.5	3.8, 3.4	−, 3.5	1, 3.2	−
		10^6	−, −	+, +	+, +	−, 0.3	+, +	NT
	48	10^5	−, −	−, −	−, −	−, −	−, −	−
		10^6	−, −	−, −	−, −	−, −	−, −	NT
	72	10^5	−, −	−, −	−, −	−, −	−, −	−
		10^6	−, −	−, −	−, 1.0	−, −	−, +	NT
	96	10^5	−, −	−, −	−, −	−, −	−, −	NT
		10^6	−, −	−, −	−, −	−, −	−, −	NT

[a] A mixture of equal amounts of CVD101 and V286 was introduced into the rabbit intestine by duodenal inoculation.
[b] Between duodenal inoculation and autopsy.
[c] Number of cells of each strain suspended in 10 ml of PBS and used for duodenal inoculation.
[d] Results for two rabbits are shown. Recovery is expressed as $\log_{10}$ number of cells detected per milliliter. +, detected after enrichment; −, not detected after enrichment; NT, not tested.
[e] Entrance to the ligated cecum.

growth of ALA auxotrophs may occur in the anaerobic compartment of the intestine (the lumen), where the persistence of the bacteria is limited by peristalsis, but not once the bacteria are attached to the intestinal epithelium, where the oxygen tension is high. In this context, it is important to note that the attachment of *V. cholerae* to the intestinal epithelium is required for the expression of clinical symptoms (10). Thus, bacteria present in the lumen are relatively harmless compared with bacteria attached to the intestinal wall. The ability of attenuated *V. cholerae* strains to grow anaerobically may be beneficial, since it may allow the strains to persist longer in the intestine and thus induce a stronger immune response.

In this study, transposon mutagenesis was used to isolate two ALA-requiring *V. cholerae* strains, designated V286 and V287. Compared with CVD101, both mutants showed diminished growth under aerobic conditions in the absence of ALA. Microaerophilic growth of both ALA auxotrophs was less affected. Southern blotting revealed that the transposon was inserted in different positions in the chromosomes of V286 and V287. As yet it is not clear whether the two strains harbor the transposon in different genes or at different positions in the same gene. Mutations in a number of genes may result in ALA auxotrophy (6). Cloning and sequencing of the loci containing the transposon will enable us to establish which genes are affected in the ALA auxotrophs.

Strain V286 was selected for further studies, because it was more severely restricted for aerobic growth than strain V287. As we had anticipated, strain V286 was able to continue growth under restrictive conditions (i.e., to coast) for a number of generations (approximately four) before growth stopped completely (Fig. 3). This coasting effect may

TABLE 3. Protection against *V. cholerae* C5 of rabbits immunized with a single dose of V286[a]

Vaccination	Culture conditions[b]	Dose[c]	Outcome of challenge[d]	Pooled results[e]	Recovery of C5[f]
PBS			4/4		++, ++, ++, ++
V286	MMak	10^5	2/0	4/0 (0.029)	−, −
		10^7	2/0		−, +
	MMk	10^3	2/0	8/0 (0.002)	−, −
		10^5	4/0 (0.029)		−, −, −, −
		10^7	2/0		−, +

[a] Rabbits were vaccinated by duodenal inoculation with V286 (or PBS). Challenge with 10^5 *V. cholerae* C5 occurred after 5 weeks.
[b] V286 was cultured in MMak and subsequently used to inoculate MMak or MMk. The latter growth condition was used to deplete cells of endogenous heme and thus prevent coasting (see Materials and Methods for details).
[c] Number of cells used for duodenal inoculation.
[d] Number of rabbits challenged/number of rabbits that developed choleralike symptoms or succumbed within 48 h. When statistically significant results were obtained ($P \leq 0.05$), P values are indicated in parentheses.
[e] As described in footnote *d*, except that results for cells grown in MMak or MMk were pooled.
[f] Recovery of challenge strain at autopsy. Results for individual rabbits (two or four per group) are shown. ++, $>10^9$ *V. cholerae* C5 per g of intestinal contents; +, *V. cholerae* C5 detected after enrichment; −, *V. cholerae* C5 not detected.

contribute to the immunogenicity of V286 by allowing it to persist longer in the intestine.

To determine whether V286 was affected in its ability to colonize the rabbit intestine, we performed in vivo competition experiments by coinfection with mutant and parental strains (Table 2). The parental strain persisted in the rabbits for up to 4 days, while the mutant strain was generally not detected more than 1 day after infection. On the first day after infection, the mutant strain was found in numbers 10^3- to 10^4-fold smaller than the parental strain, suggesting that V286 is severely impaired in its ability to colonize the rabbit intestine. These results also suggest that aerobic growth plays an important role in the colonization of the rabbit intestine by *V. cholerae*.

In contrast to CVD101, V286 could not be isolated from the feces of rabbits during the competition experiments. Thus, it seems unlikely that *V. cholerae* ALA auxotrophs will contaminate the environment or cause inadvertent infections when they are used to vaccinate humans. Furthermore, even if *V. cholerae* ALA auxotrophs remain viable after passage through the human intestinal tract, it seems unlikely that they will survive long in the environment because of their requirement for ALA.

The in vivo competition experiments clearly demonstrated that V286 was less able to colonize the intestinal tract than its parental strain. However, it is conceivable that, under conditions in which V286 does not have to compete with CVD101, it is unimpaired in its ability to colonize. Preliminary experiments in which V286 and CVD101 were used separately to infect rabbits also suggested that V286 is less able to colonize than CVD101.

Although the in vivo competition experiments suggested that V286 was defective in its ability to colonize the rabbit intestine, it was very effective in inducing protective immunity (Table 3). Even when bacteria were starved for ALA before infection to prevent coasting, a single dose was able to protect against cholera.

In conclusion, V286, an ALA auxotroph of *V. cholerae* CVD101, was less able to colonize the rabbit intestine than CVD101, displayed high vaccine efficacy, and was not shed in the environment in a viable form. ALA auxotrophs may form the basis of effective and safe live cholera vaccines.

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