The genome of *Vibrio cholerae* contains two different and functional genes for aspartate semialdehyde dehydrogenases

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Recibido: 27 de noviembre de 2002. Aceptado: 13 de diciembre de 2002.

Palabras clave: *Vibrio cholerae*, deshidrogenasa del aspartato-semialdehído, mutantes *asd*, VC2036, VC2107.
Key words: *Vibrio cholerae*, aspartate semialdehyde dehydrogenase, *asd* mutants, VC2036, VC2107.

RESUMEN. En *Vibrio cholerae* se identificaron dos genes *asd,* los cuales fueron clonados en *Escherichia coli* χ6097, por complementación del defecto de la deshidrogenasa del aspartato semialdehído (Asd). La actividad de la Asd fue también confirmada *in vitro* para cada producto génico. Se determinaron las secuencias nucleotídicas y los polipéptidos predichos, los cuales fueron comparados con las secuencias depositadas en las bases de datos *Genbank* y *Swissprot*, respectivamente. Cada producto génico pertenece a uno de los dos grupos de homología encontrados para Asd en la base de datos *Swissprot.* Los genes *asd* descritos en este trabajo corresponden a los genes VC2036 y VC2107 del cromosoma I del genoma de *V. cholerae* depositado en el Instituto de Investigaciones Genómicas (TIGR) de EE.UU. Se obtuvieron mutantes de cada gen, pero el doble mutante solo se obtuvo en presencia de un gen *asd* complementario en *trans*. Estos mutantes fueron caracterizados microbiológicamente, así como su comportamiento *in vivo* e *in vitro,* en modelos animales y bajo condiciones de laboratorio.

ABSTRACT. Two *asd* genes were identified in *Vibrio cholerae* and cloned in *E. coli* on the basis of their ability to complement the Asd defect of strain χ6097. The aspartate semialdehyde dehydrogenase activity of each gene product was also confirmed *in vitro*. Although both genes were able to complement the *asd* defect in *E. coli*, they were of different size and no important homology was found between them. The nucleotide sequences and the predicted polypeptides were established and compared with the sequences deposited in the Genbank and the Swissprot, respectively. Each gene product belongs to one of two groups of homology found for Asd in the Swissprot. The *asd* genes found in this work corresponded to genes VC2036 and VC2107 of the chromosome I of the *Vibrio cholerae* genome deposited at the TIGR. Mutants in each of these genes were obtained and characterized microbiologically, but the double mutant was only attained in the presence of a trans-complementing *asd* gene. This restriction to generate the double mutant was found to reflect the absence of an efficient mechanism for diaminopimelic acid (DAP) transport in *Vibrio cholerae*. The *in vivo* and *in vitro* behavior of the constructed mutants was also evaluated in animal models and under conditions of laboratory.

INTRODUCTION

Bacteria synthesize lysine, methionine, threonine and isoleucine from aspartate.[1] Two successive enzymatic reactions convert aspartate into aspartate semialdehyde, the precursor used for the synthesis of DAP and lysine in the lysine branch of the pathway and for the synthesis of homoserine, the common precursor of methionine, threonine and isoleucine in the two other specific branches of the pathway.

Aspartate semialdehyde dehydrogenase, encoded in the *asd* gene, is the enzyme that converts β-aspartyl phosphate into aspartate semialdehyde. The *asd* genes from several bacteria have been studied, cloned and sequenced.[1-11] According to their nucleotide sequences and predicted polypeptides, they can be classified into two groups of homology. The factors that determine which of these groups is represented in each bacteria, are not established. However, a single gene belonging to one of these families has been described in several bacterial species to date. It is yet unexplained why there is only a single aspartate semialdehyde dehydrogenase in bacteria where

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Revista CENIC Ciencias Biológicas, Vol. 35, No. 3, 2004.

this enzyme catalyses a crucial step in a biosynthetic pathway leading to four products.[1]

Previous to this work, one *asd* gene was cloned from *Vibrio cholerae* CVD101 and deposited in the GenBank under accession number X55363; however, no paper disclosing its function has appeared to date. A paper published by Karaolis *et al*., describing the sequence of this housekeeping gene in several clones of *Vibrio cholerae* emerged in 1995.[12] The authors concluded from their analysis the existence of clonal diversity in the causative agents of the sixth and seventh pandemics of cholera.[12] In this work this gene will be referred to as *asd1*.

In the forward reaction, taking place in a bacterial cell, the substrate of Asd proteins is β-aspartyl phosphate, which is reductively dephosphorylated to aspartate semialdehyde (ASA) using NADPH as cofactor.[2] The most commonly used enzymatic methods measure the dehydrogenation of ASA in the reverse reaction with NADP as cofactor.[8] No report has been found in the available literature for any Asd protein using NAD(H) as cofactor.

Mutants of several bacteria with dysfunctional *asd* genes are known to be auxotrophs for DAP, the immediate precursor to lysine in this biosynthetic pathway.[1] Diaminopimelic acid is an essential component of the microbial cell wall in Gram negative bacteria and in *Vibrio para-haemolyticus*, a near relative of *Vibrio cholerae*.[13] A bacterial cell unable to synthesize this molecule lyses when trying to grow in the presence of lysine, threonine and methionine[1] due to the lack of this compound to synthesize the rigid cell wall which otherwise would prevent the osmotic lysis. However, mutants of this gene are able to grow in LB when supplemented with DAP.

This gene has also been used to construct balanced lethal systems for expression of foreign genes in avirulent strains of *Salmonella* and *Shigella*.[4] Other genes from this branch have also been used in different microorganisms for identical purposes.

The aim of this work was to test whether the two *asd* genes present in *Vibrio cholerae* complemented the Asd defect of *E. coli* χ6097 and encoded the predicted Asd enzymatic activity. Additionally, if both genes were functional in the bacterium and if it were possible to obtain the double mutant in the presence of an exogenous source of DAP. Besides, it was investigated the contribution of each gene to the synthesis of aspartate-derived amino acids by growth of mutant and wild type bacteria in minimal media with different aminoacid supplements. The contribution of each gene to the *asd* pool of transcripts was also analyzed. Finally, it was studied whether vibrios could use DAP or lysine as nitrogen sources.

MATERIALS AND METHODS

Bacterial strains, primers, plasmids and media

The most relevant bacterial strains and plasmids used in this study are listed in Table 1. All strains were conserved frozen at –80 °C in Luria Bertani medium (LB) containing 20 % glycerol. Bacterial strains were routinely propagated at 37 °C in LB with the adequate supplements. Alternatively, they were studied in a modification of syncase medium. This minimal medium (SM) consisted of Na_2HPO_4, 0.5 %; K_2HPO_4, 0.5 %; NH_4Cl, 0.118 %; sucrose, 0.5 % as the carbon source and a supplement of mineral salts previously described.[16] SM was supplemented with different amino acids as needed. Experiments in which lysine or DAP was used as nitrogen source, NH_4Cl was omitted. Amino acids were supplemented at 50 μg/mL each, when needed. Antibiotics were added at the following concentrations: ampicillin, 100 μg/mL; polymyxin B, 13.2 μg/mL; and kanamycin, 50 μg/mL . Diaminopimelic acid was routinely supplemented at 20 μg/mL; in some experiments, the concentration was increased to 40, 100 or 1 000 μg/mL .

Primers for this study were purchased from Centro de Ingeniería Genética y Biotecnología in Havana, Cuba. Their nucleotide sequences included appropriate sites for different restriction enzymes (Table 2).

DNA techniques and analysis

The alkaline lysis method of Birnboim and Doly[17] was used to isolate plasmid DNA from bacterial strains. Transformation of *V. cholerae* strains with plasmid DNA was achieved by electroporation and suicide vectors were mobilized into *Vibrio cholerae* by conjugation. Recombinant plasmids were constructed using standard methods[18] and tested by restriction assays. DNA restriction and modification enzymes were used according to manufacturer's instructions (Böehringer Mannheim and Amersham). *V. cholerae* chromosomal DNA was prepared as described previously.[16] For Southern blots, DNA was transferred to nitrocellulose filters by an alkaline method of capillary transfer[19] and detection was performed with DIG labeled probes generated using the DNA labeling and detection kit of Böehringer Mannheim. Probes consisted of the *V. cholerae* insert present in plasmids pALTER-ASD12 for *asd1* or in pUASD2 for *asd2* (Table 1).

Cloning of *asd1* and *asd2* genes from *Vibrio cholerae*

For cloning of *asd1*, chromosomal DNA from C7258 (Table 1) and plasmid DNA of pBR322 were doubly digested with *Eco*RI and *Bam*HI, *Sal*I and *Eco*RI, *Bam*HI and *Hin*dIII, and *Eco*RI and *Hin*dIII. The corresponding ligations were set between *Vibrio* DNAs and equally digested plasmid. After reaction, each ligation was used to transform strain χ6097 by electroporation and transformants were selected in LB plates supplemented with ampicillin (LBA). Plasmid DNA purified from clones showing no requirement for DAP —expected to contain the *asd* gene— were tested again for complementation. Plasmids carried by one of these clones was denominated pBASD1 and was shown to contain a *Hin*dIII insert of 1.284 kb from *Vibrio cholerae* DNA, as determined by restriction analysis, Southern-blot and sequencing.

To clone the 5´end of *asd2*, two degenerate oligonucleotides, 4371 and 4372, were designed. The nucleotide sequence of 4371 contained a site for *Bam*HI and the nucleotide sequence of 4372 contained a site for *Pst*I at the 5' end (Table 2). These oligonucleotides were used to amplify a PCR product from total DNA of *V. cholerae* strains 638 and S12CMY12 (Table 1). The amplified PCR product from 638 was doubly digested with *Bam*HI and *Pst*I and due to the presence of an internal *Bam*HI site, it had to be cloned into *Bam*HI linearized pUC19 to obtain pUASD2. This fragment was excised, gel purified, labeled and used to probe a blot containing *Vibrio cholerae* DNA digested with different enzymes to verify the *V. cholerae* origin of the insert. The sequence of the *V. cholerae* fragment in this plasmid was later established.

Since pUASD2 contained only a minor portion of the 5´end of *asd2*, the oligonucleotides 5022 and 5023

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant properties	Reference - origin
Vibrio cholerae		
C7258	Wild type, O1, El Tor, Ogawa.	Perú, 1991. Gift of R.A.F.
638	O1, El Tor, Ogawa, Prototroph, *ΔctxF, hap::cat* from C7258.	(14)
S12CMY12	638 *Δasd1*.	This study.
638ASD2K22	638 *asd2::Km*.	This study.
E. coli		
χ6097	F⁻ *araΔ(lac-pro)* Φ80d*lacZΔM15 rpsL ΔasdA4*.	(10)
SM10(λpir)	*Thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu, Km*ʳ, *λpir*.	(14)
JM109	F' *traD36 lacI*ᵠ *Δ(lacZ)M15 proA*⁺*B*⁺/*e14(McrA⁻) Δ(lac-proAB) thi gyrA96 (Nal*ʳ*) endA1 hsdR17* ($r_K^- m_K^+$) *relA1 supE44 recA1*.	Promega.
Plasmids		
pBR322	Multipurpose cloning vector.	Biolabs.
pUC19/18	Multipurpose cloning vector.	Biolabs.
pALTER-*Ex*2ScaI	pALTER-*Ex*2 (Promega) with an 8-mer (*Eco*RI) linker inserted into the unique *Sca*I site.	This study.
pCVD442	Apʳ, *Sac*B⁺, Suicide vector for allelic exchange	(15)
pBASD1	pBR322 containing the *Vibrio cholerae asd1* as a 1.3 kb *Hin*dIII fragment.	This study.
pUASD11	pUC19 carrying the *Hin*dIII insert from pBASD1.	This study.
pALTER-ASD12	The *Hin*dIII insert of pBASD1 subcloned into pALTER-*Ex*2ScaI.	This study.
pALTER-ASD9	pALTER-ASD12 Δ(*Sca*I-*Kpn*I). Deletion of 261 bp internal to the *Vibrio cholerae asd1* (Figures 2 and 3).	This study.
pSASD12	pCVD442 bearing the internally deleted copy of *asd1* created in pALTER-ASD9.	This study.
pUASD2	The PCR fragment generated with the degenerate oligonucleotides 4371 and 4372 coding for part of the aminoterminal region of Asd2 (Fig. 5) inserted into pUC19.	This study.
p8LASD2	The PCR fragment amplified with oligonucleotides 5022 and 5023 cloned into the *Hin*dII site of pUC18 in the same direction as *lacZ* to originate the fusion protein LacZ::Asd2 represented in Fig. 1	This study.
pALASD2	The *lacZ::asd2* fusion subcloned into pALTER-*Ex*2, cloramfenicol resistant.	This study.
pU8ASD2C	The PCR fragment amplified with primers 4792 and 4791 cloned into pUC18, containing the internal fragment of *asd2*.	This study.
PQE32ASD1	The PCR fragment amplified with primers 3817 and 3818, containing the complete coding sequence of *asd1* was cloned into *Sma*I-digested pQE32 (Quiagen).	This study.
PQE31ASD2	The *Kpn*I-*Hind*III fragment from p8LASD2 cloned into pQE31 (Quiagen).	This study.

Plasmids pU8ASD21, pU8ASD2K and pCVDASD2K are described in the text.

were synthesized. The PCR product amplified from strain *Vibrio cholerae* C7258 with the primer pair and Vent Polymerase was cloned into the *Hin*dII site of pUC18 in the same direction as *lacZ* to originate the fusion protein LacZ::Asd2 (Fig. 1). This plasmid was denominated p8LASD2.

Complementation of *E.coli* χ6097 with *asd1* and *asd2* gene constructs

$CaCl_2$ competent cells of χ6097 were transformed with either pUASD11 or p8LASD2 and selected in LB plates supplemented with ampicillin. Growth indicated a complementing effect of these plasmids. This strain was also transformed with plasmids containing mutated *asd* genes (referred to as non-complementing plasmids in this paper) and selected by the antibiotic resistance marker (tetracicline, kanamycin or cloramfenicol) in the presence of DAP. To assess the complementing ability of each mutant plasmid, colonies of each transformant were inoculated into LB in the absence of DAP and incubated overnight for growth.

Sequencing of *asd1* and *asd2* genes

Double stranded plasmid DNA was sequenced by the method of Sanger,[20] using the T7-Sequenase 7-deaza-dGTP sequencing kit V2.0 (Amersham, Life Science), according to manufacturer's instructions. The strategy employed for sequencing *asd1* was based on plasmids pUASD11, pUASD11H, pUASD11K, and pUASD11P, derivatives of pUC19 containing the fragments *Hin*dIII, *Hin*dIII-*Hin*cII, *Hin*dIII-*Kpn*I, and *Hin*dIII-*Pst*I of *asd1*, respectively (Fig. 2). For sequencing of *asd2*, the plasmids pUASD2, pU8ASD2C and p8LASD2 were employed as templates (Table 1). Universal primers for pUC were used for sequencing in addition to the primers 3772, 3817, 3773, and 3818 for *asd*1 and 5022, 4704, 4703, 4791, and 5023 for *asd*2, which were also designed for double-stranded sequencing of the inserts. The nucleotide sequences were analyzed with softwares CLONE 3.11 and CLUSTAL W.[21] The BLAST

Revista CENIC Ciencias Biológicas, Vol. 35, No. 3, 2004.

Table 2. Oligonucleotides designed for the present work.

Oligonucleotide	Sequence	Orientation	Positioning*
asd1-specific			nt. 1 to 1 014.
3772	aagcttgttacccaaagcgcc	sense	-270
3773	cactggccagcaggaaaag	antisense	114
3817	agccaacaatttaatgttgcc	sense	3
3818	gcttagaagtaatcgcgtac	antisense	1 015
asd2-specific			nt. 1 to 1 113.
4371	cggatcc_gnggnatggtngg	sense	26
4372	aactgcag_acngt(g/a)ca(g/a)ttncc	antisense	407
4703	gatgctgtcattacctgc	sense	195
4704	aatttgcgatgtactgaag	antisense	117
4791	aaggcct_cacaagagctggtcg	antisense	1 058
4792	ggaattc_gtaggtttagtaggttgg	sense	7
5022	ggaattc_**atg**agagtaggtttagtag	sense	1121
5023	gggttcgaa_ggagacga**tta**tttc	antisense	

*Positioning indicates the nucleotide to which the 5´end of each primer anneals to, taking as nucleotide 1 the "A" of the start codon in each gene.
Restriction sites added at the 5´end are shown separated from the primer sequence with a underscript hyphen.
Start codon in primer 5022 and the triplet complementary to the stop codon in primer 5023 are indicated in bold face.

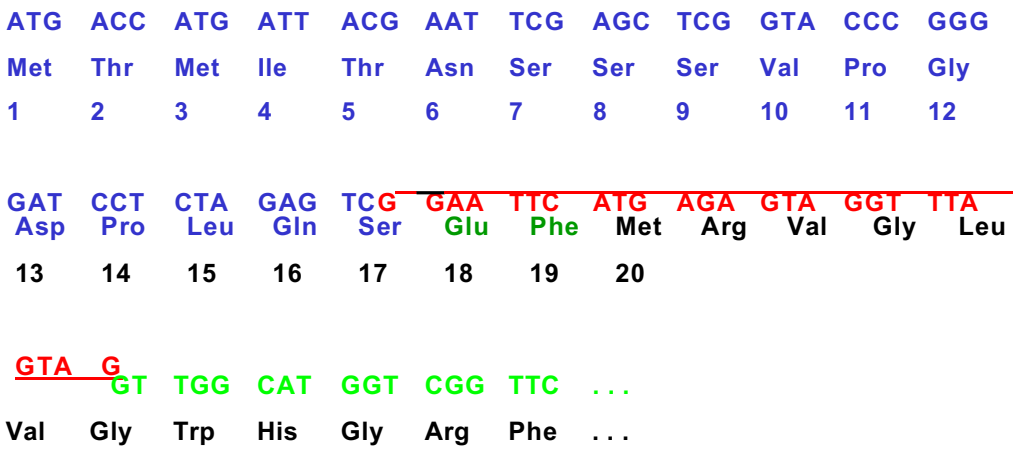

Fig. 1. *Representation of the* lacZ::asd2 *fusion construct and its deduced polypeptide encoded in p8LASD2 and in pALASD2 as corroborated by nucleotide sequencing. Only the amino terminal end is represented. This in frame fusion complements the Asd defect of* E. coli *χ6097 driven by the* lacZ *promoter of pUC18. This fusion adds 19 amino acids to the N-terminal of Asd2 from* Vibrio cholerae. *The first 17 amino acids represented in the figure come from the* E. coli *LacZ; the next two amino acids were introduced by the first nucleotides of the primer, and methionine 20 corresponds to the first amino acid of Asd2. The nucleotide sequence corresponding to primer 5022 is underlined.*

software[22] was used for protein alignment and comparisons.

Construction of *asd1* and *asd2* mutants of *Vibrio cholerae*

The wild type *asd1* or *asd2* gene of *Vibrio cholerae* 638 was replaced with a mutated allele by a methodology of allelic exchange previously described.[16]

A suicide vector containing a mutated *asd1* was constructed in several steps. First, the *Kpn*I/*Sca*I fragment was deleted from the insert present in plasmid pALTER-ASD12 (Table 1 and Fig. 2) to obtain pALTER-ASD9, which was unable to complement the Asd defect in *E. coli* χ6097. The *Xba*I fragment from pALTER-ASD9 (Fig. 2), containing the inactivated gene was excised and cloned into the unique *Xba*I site of pCVD442 to obtain the suicide plasmid pSASD12.

A suicide vector containing a mutated *asd2* was also constructed in several steps. Plasmid pU8ASD21 was first constructed. It was obtained after cloning the internal fragment of *asd2*, amplified by PCR from *Vibrio cholerae* C7258 with the primers 4792 and 4791 as an *Eco*RI-*Stu*I fragment into pUC18 digested with *Eco*RI and *Hin*dII. This plasmid was linearized with *Eco*RI,

blunted with klenow and a *Xba*I octameric linker was inserted to obtain pU8ASD2C. The kanamycin resistance determinant of pUC4K (Amershan-Pharmacia-Biotech) was inserted into the single *Bam*HI site of *asd*2 in pU8ASD2C to obtain pU8ASD2K. The *Xba*I-*Sph*I fragment of this plasmid containing the *asd2::Km* gene was transferred into pCVD442 to obtain the suicide vector pCVDASD2K, which was used to replace the *asd2* gene in *Vibrio cholerae* 638.

Each suicide plasmid was independently mobilized into *Vibrio cholerae* 638 from SM10λpir to obtain the respective ampicillin resistant cointegrate in the correspondent *asd* chromosomal locus. Correct co-integrates were selected after screening the exconjugants by Southern blot with the specific *asd* probe generated from the insert in pALTERASD12 (*asd1*) or in pUASD2 (*asd2*). Bacterial cells from three ampicillin resistant colonies were allowed to segregate in LB supplemented with diaminopimelic acid in the absence of antibiotic and plated on the same media containing sucrose at 5 %. Cells from ten sucrose-resistant ampicillin-sensitive colonies were immediately streaked, conserved and analyzed for the structure of the *asd* locus. Several clones were ob-

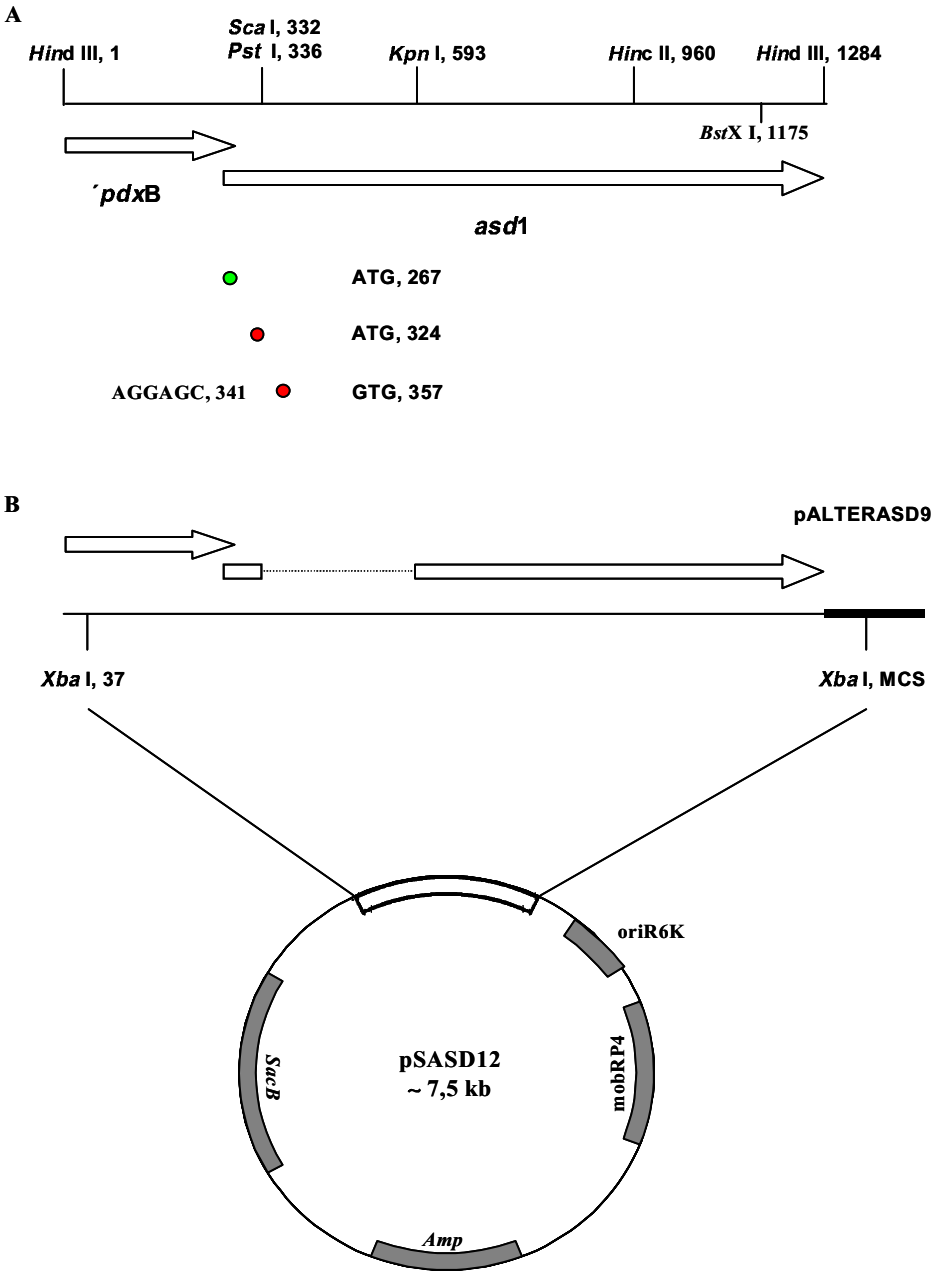

Fig. 2. *(A) Schematic representation of important features of the* Hind*III fragment containing the* asd1 *gene of* Vibrio cholerae. *Plasmid constructs used for sequencing had in common the left end* Hind*III site represented in the figure. Three putative start codons were indicated with circles accompanied by their nucleotide numbers under the respective positions. The sequence of the putative ribosomal binding site at nucleotide 341 is also represented. (B) Construction of pALTER-ASD9 and pSASD12 is represented as well as important restriction sites and their positioning. The deletion performed to obtain pALTER-ASD9 and later subcloned into pSASD12 appears represented by the dashed line. Genes are represented with arrows.*

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tained in which the wild type copy of the gene had been replaced by the mutated allele and one, designated S12CMY12 (*asd1* mutant) and other designated 638ASD2K22 (*asd2* mutant), were subsequently characterized.

ASD activity determination

For determination of the enzymatic activity of Asd, the substrate aspartate semialdehyde was chemically synthesized by ozonolysis of allylglycine at 0 °C . Briefly, 20 mmol of the reactant were dissolved in 20 mL of 1 mol/L HCl and a current of ozone was passed through the solution until cessation of ozone consumption, as determined spectrophotometrically.[23] The product obtained was used without further purification as a substrate in aspartate semialdehyde dehydrogenase determination.

The enzymatic assay for determination of aspartate semialdehyde dehydrogenase was done in the reverse reaction and was set in 1 mL of diethanolamine-HCl buffer 0.03 mol/L containing NAD^+ or $NADP^+$ (0.8 mmol/L), Na_2HAsO_4 (40 mmol/L), NaCl (120 mmol/L) and a sufficient quantity of protein. The reaction was started by the addition of 0.1 mL of 3 mmol/L substrate previously diluted and neutralized with potassium hydrogen carbonate. The rate of NAD or NADP reduction was followed spectrofotometrically at 340 nm and results were reported in units of enzyme activity. One unit corresponds to the quantity of enzyme that transform 1 μmol of NAD or NADP in one minute.

Cellular extracts for ASA dehydrogenase assays of Asd1 were obtained by successive vortexing of the bacterial pellet of χ6097 transformed with pQE32ASD1 (50 mL of culture) with glass beads in 1 mL of PBS followed by a centrifugation step to clear the extract from cellular debris and exhaustive dialysis against PBS. Protein concentration was adjusted to 0.5 mg/mL and immediately used for the assays. The his-6 tagged Asd2 was purified from cellular extracts of *E. coli* XL-1 (pQE31ASD2), using the his-trap kit, as described by the manufacturer (Amershan-Pharmacia-Biotech). Protein concentration was adjusted to 0.1 mg/mL and analyzed for ASA dehydrogenase activity.

Northern blot

For Northern blot, total RNA was purified from mid-log phase cultures of *Vibrio cholerae* C7258 growing either in LB or in SM minimal media. RNA was quantitated spectrophotometrically and equivalent quantities were denatured in the presence of formamide and formaldehyde at 60 °C. Duplicate samples were loaded into parallel agarose gels containing formaldehyde and run at 40 V during 4 h. RNA messages sized in each gel were transferred to nitrocellulose filters by the alkaline capillary transfer technique of Chomczynski.[19] One of the filters was analyzed with an *asd1* specific probe and the other with an *asd*2 specific probe. Probes were the same as described for Southern blots but were labeled with (α-P^{32}) dATP.

Characteristics of growth of S12CM Y12 and 638ASD2K22

The kinetics of growth was analyzed for *asd* mutants of *Vibrio cholerae* growing in rich media and in minimal media or in minimal media supplemented with different amino acids. For determinations of growth rates in rich media, strains were grown overnight in LB, diluted 1:200 in 75 mL of fresh broth and followed spectrophotometrically until stationary phase. For determinations of growth rates in minimal media, bacterial strains were previously adapted to the medium of the assay by a round of subculture in SM supplemented with 1 % casaminoacids followed by a round of growth in SM. Bacterial strains treated like this were harvested, washed in PBS, inoculated into fresh SM media and the absorbance at 600 nm was followed until stationary phase.

Colonizing capacity of *Vibrio cholerae* mutants

The colonizing capacity of strains S12CMY12 and 638ASD2K22 was evaluated in the infant mouse model of cholera as previously described.[16]

Genbank deposits

The sequence corresponding to *asd1* and *asd2* were deposited in the EMBL database under the accession numbers Y15281 and AY035394, respectively. The sequence of *asd1* in *Vibrio cholerae* C7258 was 99 % identical to the sequence of VC2107 in the genome of *Vibrio cholerae* N16961. The sequence of *asd2* was 99 % identical to the sequence of gene VC2036 in the genome of *Vibrio cholerae* N16961.

RESULTS

Cloning of *asd1* gene from *Vibrio cholerae*

Plasmid pBASD1 contains a *Hin*dIII insert of ~1.3 kb from *V. cholerae* which is able to complement the *E. coli asd* mutant χ6097.

The insert present on this plasmid was subcloned into pUC19 and the nucleotide sequence was established and deposited in the EMBL databank under the accession number Y15281. A detailed analysis of this sequence revealed the existence of a complete open reading frame from nucleotide (nt.) 258 trough nt. 1280 which was assigned the *asd1* coding function (Fig. 3). One out of three putative start codons within this orf, ^{267}ATG, ^{324}ATG or ^{357}GTG is used by *V. cholerae* to initiate the synthesis of the Asd1 peptide (Fig. 2 and Fig. 3). The presence of the hexanucleotide AGGAGC that almost perfectly matches the consensus for an *E. coli* ribosomal binding site, located 12 nt. upstream from ^{357}GTG, prompted us to examine whether it was the start or an internal *asd1* codon. The insertion of a *Sal*I octameric linker into the *Sca*I restriction site at nt. 332 abolished the complementing ability of this fragment; so the ^{357}GTG was not the initiation codon but an internal triplet of *asd1*. Consequently either ^{267}ATG or ^{324}ATG correspond to the *asd1* start codon. For practical reasons it was not experimentally determine which of them was functioning as such and it was assumed that ^{267}ATG codes for the N-terminal methionine of the protein. According to this assumption, the *Vibrio cholerae* Asd1 protein is predicted to be a polypeptide of 337 amino acids with a calculated molecular weight of 37.4 kDa.

The deduced amino acid sequence of this protein was compared to protein sequences in the Swissprot database. It shared high percents of identity with the Asd proteins of *Vibrio cholerae* strain CVD101 (99 %), *Vibrio mimicus* (98 %), *Shewanella violacea* (68 %), *Shewanella* sp (68 %), *Legionella pneumophila* (60 %) and *Aquifex aeolicus* (54 %). The identity is well distributed through the entire peptide and proteins are about the same size. Also Usg1 protein from *E. coli* displayed important homology through the complete polypeptide, being 65 % similar and 48 % identical to the queried sequence. It was also homologous to Asd proteins of *Bacillus subtilis*, *Mycobacterium smegmatis*, and

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     S  L  L  P  K  A  P  V  P  K  V  Y  L  E  R  A  W  D  E  E
  1 AAGCTTGTTACCCAAAGCGCCTGTGCCCAAAGTTTATCTAGAGCGAGCATGGGACGAGGA

     T  L  R  T  L  T  Q  I  I  Y  D  V  R  K  D  D  A  Q  F  R
 61 AACACTGCGTACGCTAACTCAAATTATTTATGATGTGCGTAAAGATGACGCACAGTTTCG

     R  E  I  H  Q  P  G  A  F  D  L  M  R  K  H  Y  W  D  R  R
121 CCGCGAGATTCACCAACCCGGAGCGTTTGACTTGATGCGCAAACACTATTGGGATAGACG

     E  Y  S  A  V  T  L  A  G  G  A  D  C  H  L  A  P  L  A  K
181 TGAATACAGCGCGGTGACTCTAGCTGGAGGCGCTGATTGCCATTTGGCTCCTTTGGCTAA

     L  G  F  Q  V  E  V  C  D  E  P  T  I  ***
                                  M  S  Q  Q  F  N  V  A  I  F  G
241 ACTCGGTTTTCAAGTAGAGGTATGTGATGAGCCAACAATTTAATGTTGCCATTTTTGGCG

    A  T  G  A  V  G  E  T  M  L  E  V  L  Q  E  R  E  F  P  V
301 CAACCGGCGCTGTAGGCGAAACCATGTTGGAAGTACTGCAGGAGCGTGAGTTTCCTGTGG
                                 ScaI
    D  E  L  F  L  L  A  S  D  R  S  E  G  K  T  Y  R  F  N  G
361 ATGAACTTTTCCTGCTGGCCAGTGACCGCAGTGAGGGAAAAACCTATCGCTTCAACGGTA

    K  T  V  R  V  Q  N  V  E  E  F  D  W  S  Q  V  H  I  A  L
421 AAACCGTGCGTGTGCAAAATGTCGAAGAATTTGACTGGTCGCAAGTGCATATTGCGCTGT

    F  S  A  G  G  E  L  S  A  K  W  A  P  I  A  A  E  A  G  V
481 TTTCTGCCGGTGGTGAGCTTTCTGCCAAGTGGGCTCCGATTGCCGCAGAAGCGGGTGTTG

    V  V  I  D  N  T  S  H  F  R  Y  D  Y  D  I  P  L  V  V  P
541 TGGTGATCGACAACACTTCACACTTCCGTTACGACTACGACATTCCTCTTGTGGTACCTG
                                                      KpnI
    E  V  N  P  E  A  I  A  E  F  R  N  R  N  I  I  A  N  P  N
601 AAGTGAACCCAGAGGCGATTGCAGAATTTCGTAACCGCAATATTATTGCTAACCCAAACT

    C  S  T  I  Q  M  L  V  A  L  K  P  I  Y  D  A  V  G  I  E
661 GCTCAACCATTCAGATGCTTGTCGCGCTAAAGCCGATTTACGATGCGGTGGGGATTGAAC

    R  I  N  V  T  T  Y  Q  S  V  S  G  A  G  K  A  G  I  D  E
721 GCATTAACGTGACGACTTACCAATCGGTCTCAGGTGCTGGGAAAGCTGGGATTGATGAGT

    L  A  G  Q  T  A  K  L  L  N  G  Y  P  A  E  T  N  T  F  S
781 TAGCAGGACAAACCGCTAAACTGCTCAATGGCTATCCAGCGGAAACGAATACTTTTAGCC

    Q  Q  I  A  F  N  C  I  P  Q  I  D  Q  F  M  D  N  G  Y  T
841 AGCAGATCGCGTTTAACTGTATTCCACAAATCGATCAGTTTATGGACAACGGTTATACCA

    K  E  E  M  K  M  V  W  E  T  Q  K  I  F  N  D  P  S  I  M
901 AAGAAGAGATGAAGATGGTGTGGGAAACCCAGAAAATCTTCAATGATCCTTCGATCATGG

    V  N  P  T  C  V  R  V  P  V  F  Y  G  H  A  E  A  V  H  V
961 TCAACCCAACGTGCGTACGTGTACCGGTATTCTACGGTCATGCGGAAGCCGTACACGTGG

    E  T  R  A  P  I  D  A  E  Q  V  M  D  M  L  E  Q  T  D  G
1021 AAACTCGCGCGCCAATTGATGCTGAGCAAGTGATGGATATGCTAGAGCAAACCGATGGTA

    I  E  L  F  R  G  A  D  F  P  T  Q  V  R  D  A  G  G  K  D
1081 TTGAACTGTTCCGCGGTGCGGATTTCCCAACTCAAGTGCGTGATGCCGGCGGTAAAGACC

    H  V  L  V  G  R  V  R  N  D  I  S  H  H  S  G  I  N  L  W
1141 ATGTGTTGGTTGGGCGTGTACGAAATGACATTAGCCACCATAGTGGAATCAACTTGTGGG

    V  V  A  D  N  V  R  K  G  A  A  T  N  A  V  Q  I  A  E  L
1201 TAGTGGCGGATAACGTGCGTAAAGGTGCTGCCACCAATGCGGTGCAAATTGCAGAGCTGC

    L  V  R  D  Y  F  ***
1261 TCGTACGCGATTACTTCTAAGCTT
```

Fig. 3. *Nucleotide sequence and predicted polypeptides encoded in the 1.3 kb* Hind*III fragment cloned in pBASD1. Amino acids are represented in the single letter code and above the nucleotide sequence. Stop codons appear overscored with three asterisks. The partial orf represented at the 5´ end corresponds to* pdxB *and the downstream gene corresponds to* asd1. *No homologues to bacterial promoters were found within the fragment. Codons* ^{267}*ATG,* ^{324}*ATG and* ^{357}*GTG are underlined. The restriction sites for* Sca*I and* Kpn*I used to inactivate* asd1 *are indicated.*

Streptococcus mutans. The above evidences support the function attributed to this coding sequence.

Caracterization of *asd1* locus and its 5´ flank

The structure of the *asd1* locus of *Vibrio cholerae* was analyzed by Southern blot. A DIG labeled *asd1* specific probe was prepared from the 1.283 kb *Hin*dIII fragment, employing the DIG DNA labeling and detection kit from Böehringer Manheim. Appropriately digested DNAs were electrophoresed, blotted, and probed with the *asd1* specific probe. Several restriction enzymes tested, which did not cut inside *asd1*, generated a single *asd1* specific fragment, suggesting the existence of a single copy of this gene in the genome of *V. cholerae*.

To define which gene flanked *asd1* by the 5´ end, the nucleotide sequence was examined (Fig. 3). An

incomplete orf was found extending from the 5' end of the 1,283 kb fragment (Figures 2 and 3), to nt. 280 located in *asd1*. The predicted polypeptide encoded in this orf was blasted against the Swissprot and found to be homologous to the *E. coli* PdxB polypeptide. Next, it was blasted the TIGR database of the N16961 genome of *V. cholerae* with the *E. coli pdxB* and *V. cholerae asd1* genes. The results demonstrated that a gene homologous to *E. coli pdxB*, effectively lies 5' to *asd1* in the chromosome of *V. cholerae* N16961 whose sequence is deposited at the TIGR (http://www.tigr.org) and in the chromosome of C7258 whose sequence is the subject of this article.

PdxB in *E. coli* functions as 4-phosphoerythronate dehydrogenase and is essential in the route for the biosynthesis of vitamin B6.The association found between these two genes, in which *pdxB* overlaps *asd1*, is suggestive of the existence of a single transcription unit for their expression.

Construction and characterization of a *Vibrio cholerae asd1* deletion mutant

The *asd1* gene of strain S12CMY12 has an internal deletion of the 261 bp formerly comprised between the *Sca*I and *Kpn*I sites of the wild type gene (Fig. 3). This deletion was demonstrated to inactivate the complementing ability of plasmid pALTER-ASD12 in *E. coli*. Panel A (Fig. 4) demonstrates that clone S12CMY12 (lanes b and c) contains *asd1* in an *Nco*I fragment of about 4 kb, which is 261 bp smaller than the correspondent locus in 638 (lane d). The size of this fragment in S12CMY12 is also smaller than in the cointegrate CMY12 (lane e).

The mutation introduced into S12CMY12 was also characterized by PCR with oligonucleotides 3772 and 3818, which hybridize at the 5´and 3´ends of *asd1* (Table 2). While the PCR product from 638 was about 1.3 kb, S12CMY12 produced a single band of about 1.0 kb and the pair of primers 3772 and 3773, of which 3773 hybridizes internal to the deleted fragment, failed to amplify a product from S12CMY12.

Despite having mutated *asd1*, this strain continued being prototrophic in SM minimal plates, indicating the existence of a second gene coding for an aspartate semialdehyde dehydrogenase in *Vibrio cholerae*. This suspected gene was named *asd2*.

Cloning *asd2* from *V. cholerae*

As it was not possible to clone *asd2* by complementation in *E. coli* χ6097 with a genomic library, it was tried to amplify an internal fragment of this gene by PCR. Two degenerate primers were designed to amplify an N-terminal fragment of *asd2*. First, all *asd* deduced peptides deposited in the GenBank were downloaded and aligned using ClustalW software. After alignment, two groups of homology were found. Group 1 included Asd peptides from *Vibrio cholerae* strain CVD101, *Vibrio mimicus*, *Shewanella violaceae*, *Shewanella sp*, *Legionella pneumophila*, *Mycobacteria*, *Leptospira*, and *Streptomyces*. Group 2 included *asd* peptides from *E. coli*, *Haemophilus Influenzae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Bordetella pertussis*, *Azotovacter vinelandii* and *Actinobacillus pleuropneumoniae*. As the group 1 was represented by *asd1* in *Vibrio cholerae*, degenerate primers were designed for conserved sequences of Asd2.

The oligonucleotides 4371 and 4372 (Table 2) were used to amplify a 400-bp *asd*2 specific product from *Vibrio cholerae*, strain 638. The amplified product, presumably the coding region for the amino terminal portion of Asd2, was gel purified, labeled and used to probe a Southern Blot containing DNA from 638. This fragment hybridized to single restriction fragments of *Pst*I, *Xho*I, *Hin*dIII or *Eco*RI restricted DNA; indicating that 638 contains the DNA sequence that supported the amplification of the PCR product. The sequence of this fragment was established and shown to contain no stop codons in one of the possible orfs. The predicted amino acid sequence encoded in this orf was significantly homologous to the amino terminal fragment of Asd proteins from different bacteria of the homology group 2. They included *E. coli* (62 % identity), *P. aeruginosa* (59 % identity), and *S. thyphimurium* (60 % identity). Alignment between sequences occurred at the expected residues according to the designed primers (Fig. 5).

This fragment is represented in the genome of *Vibrio cholerae* N16961 deposited at the TIGR with no changes in single nucleotides. This result permitted to construct a hybrid sequence between this short *asd2* fragment sequenced herein and the *E. coli asd* gene and blast the resultant molecule against the N16961 unfinished genome. The extensive homology of this hybrid molecule with *asd2* in the genome of N16961 allowed to confirm the prediction on the existence of two *asd* genes in *Vibrio cholerae* and download the sequence of practically the complete coding sequence before it was available to the public. Asd2 seems to be responsible for the aspartate semialdehyde dehydrogenase that sustents prototrophic growth of S12CMY12, the *asd1* mu-

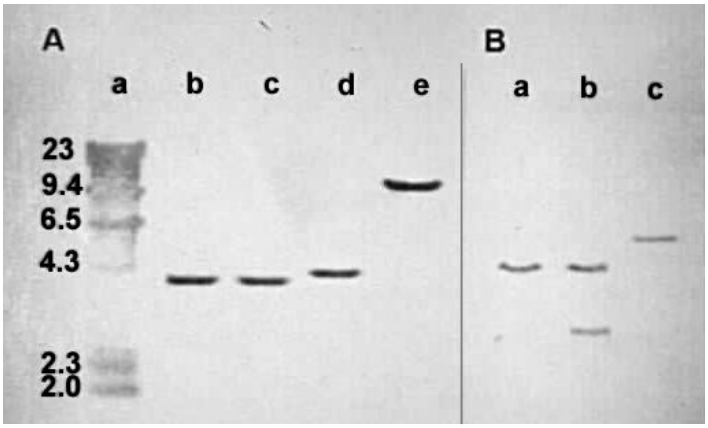

Fig. 4. *Southern blots showing the genetic structure of the* asd1 *and* asd2 *locus of mutants S12CMY12 (panel A) and 638ASD2K22 (panel B), respectively. Panel A was hybridized with the* asd1 *specific probe and panel B was hybridized with the* asd2 *specific probe. Panel (A): lane a, molecular weight marker –*Hind*III digested lambda DNA–; lane b and c,* Nco*I digested DNA of S12CMY12; lane d,* Nco*I digested DNA of 638; lane e,* Nco*I digested DNA of CMY12. Panel (B): lane a,* Eco*RI digested DNA of 638; lane b,* Eco*RI digested DNA of cointegrate 638ASD2K and lane c,* Eco*RI digested DNA of 638ASD2K22. Sizes of the different fragments of the Molecular weight marker are indicated at left in kb.*

```
E. coli              -MKNVGFIGW RGMVG SVLMQRMVEERDFDAIRPVFFSTSQLGQAAPSFGGT-TGTLQDAF
B. Pertussis         MTQAVGLVGW RGMVG SVLMQRMRDENDFALIEPVFFSTSNAG-GAAPAWAEGAGSLQNAY
H. influenzae        -MKNVGFIGW RGMVG SVLMDRMSQENDFENLNPVFFTTSQAGQKAPVFGGKDAGDLKSAF
P. aeruginosa        -MKRVGLIGW RGMVG SVLIQRMLEERDFDLIEPVFFTTSNVG-AQAPEVDKDIAPLKDAY
A. vinelandii        -MKRVGLIGW RGMVG SVLMQRMLEERDFDLIEPVFFTTSSVG-GQG-AIGKETVPLKDAY
S. typhimurium       -MKNVGFIGW RGMVG SVLMQRMVEERDFDAIRPVFFSTSQFGQAAPTFGDTSTGTLQDAF
A. pleuropneumoniae -MQNVGFIGW RGMVG SVLMDRMVQENDFANINPIFFTTSQAGQKAPVFAGKDAGELKNAF

V. cholerae              Primer 4371              FDLIEPVFFSTSQIG-VPAPNFGKDAGMLHDAF
                                                  *  . *.**.**  *             *. *.

E. coli              DLEALKALDIIVTCQGGDYTNEIYPKLRESGWQGYWIDAASSLRMKDDAIII LD PVNQDV
B. Pertussis         DIDALKKLPIIVTAQGGDYTSEVYPKLRGAGWQGIWIDAASTLRMADDAIIV LD PVNRPV
H. influenzae        DIEELKKLDIIVTCQGGDYTNEVYPKLKATGWDGYWVDAASALRMKDDAIIV LD PVNQHV
P. aeruginosa        SIDELKTLDVILTCQGGDYTSEVFPKLREAGWQGYWIDAASSLRMEDDAVIV LD PVNRKV
A. vinelandii        SIEELKSLDAIITCQGGDYTSEVFPKLRDAGWQGYWIDAASSLRMADDAVIV LD PVNRRV
S. typhimurium       DLDALKALDIIVTCQGGDYTNEIYPKLRESGWQGYWIDAASTLRMKDDAIII LD PVNQDV
A. pleuropneumoniae DIEELKKLDIIVTCQGGDYTNEVYPKLKATGWNGYWIDAASALRMEKDAIIV LD PVNQHV

V. cholerae          DIESLKQLDAVITCQGGSYTEKVYPALRQAGWKGYWI                BamHI
                      .. ** *  ..*.*** **  ..* *. .** * *.

E. coli              ITDGLNNGIRTFVG GNCTV SLMLMSLGGLFANDLVDWVSVATYQAASGGGARHMRELLTQ
B. Pertussis         IDAALKRGVRNFVG GNCTV SCMLMGLAGLFNNDLVEWMSSMTYQAASGGGAQHMRELLTQ
H. influenzae        ISEGLKKGIKTFVG GNCTV SLMLMAIGGLFEKDLVEWISVATYQAASGAGAKNMRELLSQ
P. aeruginosa        IDQALDAGTRNYIG GNCTV SLMLMALGGLFDAGLVEWMSAMTYQAASGAGAQNMRDLLKQ
A. vinelandii        IDQSLDAGVKNYIG GNCTV SLMLMALGRLRGR-PGRLDERHDLSGRFRAGAQNMRELIRQ
S. typhimurium       ITDGLNNGVKTFVG GNCTV SLMLMSLGGLFAHNLVDWVSVATYQAASGGGARHMRELLTQ
A. pleuropneumoniae ISEGLKNGIKTFVG GNCTV SLMLMAIGGLFEKDLVEWVSVATYQAASGAGAKNMRELLSQ

V. cholerae                Primer 4372


Primer 4371: cggatcc_gnggnatggtngg

Primer 4372: aactgcag_acngt(g/a)ca(g/a)ttncc
```

Fig. 5. *Protein alignment of the amino terminal portion of Asd2 from different bacteria. The deduced sequence of the internal fragment of Asd2 cloned in pUASD2 is also represented. Identical amino acids are indicated with asterisks and conserved or similar amino acids are indicated with dots. Conserved sequences in the flanks that were used for primer design are underlined. The amino acids that accommodated the internal* Bam*H I site of* asd2 *are indicated in bold face. The nucleotide sequences of the PCR primers used to amplify the internal fragment of* asd2 *are represented at the bottom of the figure.*

tant described above. According to the downloaded sequence of *asd2*, two primers were designed to amplify the complete coding sequence, establish the sequence of this gene in *Vibrio cholerae* C7258, demonstrate its complementing ability in the *E. coli asd* mutant χ6097 and construct an *asd2* mutant in *Vibrio cholerae* 638.

Both, the nucleotide sequence deposited at the TIGR genomic database and the sequence determined in this study predict a polypeptide of 370 amino acids for Asd2, which is 33 residues larger than Asd1. Nor the nucleotide sequences neither the encoded polypeptides of *asd1* and *asd2* showed significant homology between them.

Vibrio cholerae asd2 complements the auxotrophy of *E. coli* χ6097

Although it was unable to clone *asd2* by complementation in *E. coli* χ6097 from a genomic library, it was demonstrated the ability of this gene to complement the Asd defect. In doing this, plasmid p8LASD2 (Fig. 1 and Table 1) was transferred into *E. coli* χ6097 by electroporation and selected in the presence of ampicillin in LB media supplemented with IPTG in the absence of DAP. Growth of transformants indicated the complementing effect of the plasmid in *E. coli*. The presence of p8LASD2 was confirmed by restriction analysis of the plasmid contained within grown bacteria. From this observation it was concluded that *asd2* from *Vibrio cholerae* complemented the defect in *E. coli* χ6097.

In vitro enzymatic activity of Asd1 and Asd2

The enzyme activity of the *asd1* product was evaluated in extracts of *E. coli* χ6097 transformed with pQE32ASD1 (Table 1), taking parallel controls of the untransformed strain. This plasmid complemented *E. coli* χ6097. The enzyme activity was evaluated with NAD or NADP. ASA dehydrogenase activity in extracts of the transformants was detected [(0.24 ± 0.1) U/mL] in the presence of NAD but not in the presence of

NADP, while extracts of the control strain *E. coli* χ6097 did not display this activity with either NAD or NADP. The reactions did not proceed when ASA was excluded from the reactants.

The enzyme activity of the *asd2* gene product was evaluated either in extracts of *E. coli* XL-1 transformed with pQE31ASD2 or in fractions of the partially purified His(6)-Asd2 encoded in this plasmid (Table 1). ASA dehydrogenase activity was detected [(2.4 ± 0.4) U/mL] for the partially purified enzyme in the presence of NADP but not in the presence of NAD. The reactions did not proceed when the extracts were incubated with NADP in the absence of ASA.

These results clearly indicate that Asd1 is dependent on NAD while Asd2 is dependent on NADP and provide the needed support to affirm that both genes code for aspartate semialdehyde dehydrogenase activity in *Vibrio cholerae*.

Construction and characterization of 638ASD2K22

The *asd2* gene of 638ASD2K22 is insertionally inactivated with the kanamycin resistance determinant from plasmid pUC4K. This mutant was generated by allelic replacement using a pCVD442 derivative containing the insertionally inactivated gene and later characterized by Southern blot with an *asd2* specific probe (Fig. 4, panel B). This Figure shows that 638 has its *asd2* gene in a single *Eco*RI fragment of ~4.3 kb (lane a); while 638ASD2K22 has its *asd2* allele in a homologous fragment 1.3 kb higher (lane c). This is in correspondence with the insertion of the kanamycin resistance gene into *asd2*.

Like the *asd1* mutant, this strain was also analyzed for its capacity to grow in LB in the absence of DAP. As it was expected from the anticipated knowledge of the existence of *asd1*, 638ASD2K22 was able to grow in LB plates in the absence of DAP supplements. This strain was also able of prototrophic growth in minimal media. These results pointed to the functionality of *asd1* in *Vibrio cholerae*.

Kinetics of growth of *asd* mutants of *Vibrio cholerae*

The growth characteristics of 638ASD2K22 and S12CMY12 strains were analyzed in LB, in minimal media and in minimal media supplemented with different amino acid mixes. The mean generation time of each strain under each condition was determined (Table 3). The rate of growth for both mutants in LB was undistinguishable from the rate of growth of the parental strain 638. However, 638ASD2K22 grew at a significant slower rate than S12CMY12 or 638 in minimal media, being the rate of growth for S12CMY12 and 638 undistinguishable. Transformation of 638ASD2K22 with plasmid p8LASD2 restored the rate of growth to wild type levels in minimal media. This result excluded out that other mutation different from the insertional inactivation of *asd2* was responsible for the change in the rate of growth. As well, supplementation of minimal media with casaminoacids or with the end products of the aspartate pathway (threonine plus lysine plus methionine plus isoleucine -TLMI) restored the rate of growth of 638ASD2K22 to the wild type level. In contrast, supplementation with all other amino acids, except TLMI to minimal media did not restore the wild type rate of bacterial growth. As this strain was growing in minimal media at the expense of *asd1*, the results suggested that the *asd1* gene expressed lower levels of Asd1 protein in the bacteria in the conditions of the assay. Alternatively they could indicate that Asd1 is less efficient in performing the aspartate semialdehyde dehydrogenase function in *Vibrio cholerae* than the gene or the gene product of *asd2*.

DAP is the immediate precursor to lysine and an essential precursor of the peptidoglycan synthesized by several bacteria. With this element into account, it was studied if it were possible to replace lysine with DAP in the TLMI supplement needed to attain the wild type rate of growth in 638ASD2K22. No differences were found between cultures in which lysine was replaced with DAP (5 or 100 mg/mL) and those in which lysine was omitted. In neither of the two cultures the wild type rate of growth was attained, probably indicating that vibrio "ignored" the DAP present in the culture media.

Expression of *asd* genes under different culture conditions

A Northern blot was performed to screen for transcription of these two genes in wild type *Vibrio cholerae* grown under two different culture conditions: rich and minimal medium. The maximal contribution to *asd* specific transcripts was found for the *asd2* gene with a stronger contribution in minimal media (Fig. 6). The contribution of *asd1* to the pool of transcripts was negligible in rich media and stronger in minimal media but still smaller than the contribution of *asd2*. This finding suggests *asd2* as the responsible for most of the ASD activity produced in wild type bacteria under laboratory conditions.

With the *asd2* specific probe, transcripts of about 1.5 kb were observed. These transcripts perfectly accommodate the 1110 nucleotides long *asd2* orf. This indicates that *asd2* is transcribed as a single transcription unit. In fact, according to the genomic data deposited at the TIGR, the gene located upstream

Table 3. Mean generation time of *Vibrio cholerae* strains having wild type and mutant *asd* genes under different conditions of growth.

Conditionof growth	Mean generation time of strain (min)		
	638 wild type *asd*$^+$	S12CMY12 *asd1* mutant	638ASD2K22 *asd2* mutant
LB	20,9 ± 0,9	21,1 ± 1,3	22,5 ± 0,6
SM	45,0 ± 4,0	46,0 ± 4,0	103 ± 5,8
SM + CAA			48,1 ± 2,0
SM + TLMI			45,6 ± 4,0
SM + TMI			77,0 ± 3,0
SM + TMI + DAP			75,0 ± 5,0
SM + all aa., except TLMI			110 ± 4,0
p8LASD2 (SM)			41,0 ± 2,0

aa. Aminoacids. LB Luria broth. SM minimal medium. DAP diaminopimelic acid. TLMI Treonine + lysine + methionine + isoleucine, 50 mg/mL each. TMI Threonine + methionine + isoleucine, 50 mg/mL each. CAA Casaminoacids 1 %. p8LASD2 (SM) Transformed with plasmid p8LASD2 and grown in SM. Blank spaces correspond to experiences not performed.

from *asd2* runs divergently and the downstream gene runs in the opposite direction.

With the *asd1* specific probe, transcripts of more than one size were detected in minimal media grown bacteria (Fig. 6). Only transcripts over 1.0 kb can accommodate the 1014 nucleotides orf of *asd1*. A total of three transcripts longer than 1.0 kb were detected; they were ~1.5 kb, ~ 2.5 kb and > 2.9 kb long. The ~ 1.5 kb long transcript can only accommodate *asd1*. The 2.5 kb can accommodate *asd1* plus the immediately upstream gene VC2108; as they together need 2.2 kb. The > 2.9 kb can accomodate *asd1* plus VC2108 plus the immediately upstream VC2109, as they together need 3.4 kb. This is consistent with the idea that *asd1* is transcribed from its own promoter (monocistronic RNA), as well as from the promoters of VC2108 and VC2109, bicistronic and tricistronic RNAs, respectively.

Obtaining the double mutant in *asd1* and *asd2*

Mutant S12CMY12 has a dysfunctional *asd1*, while mutant 638ASD2K22 has a dysfunctional *asd2*. These two strains were used as starting strains when attempting to obtain a mutant in the counterpart *asd*. In neither of the cases the double mutant was found, not even in the presence of 1 mg/mL of DAP. It was presumed that at least one of these two genes needed to be functional in *Vibrio cholerae*. Under this assumption, a cointegrate of 638ASD2K22 with pSASD12 was obtained and denominated IJUL13. IJUL13 was transformed with the replicative plasmid pALASD2 and selected in the presence of ampicillin and cloramfenicol. The cointegrate was allowed to segregate in the presence of cloramfenicol to select for the replicative plasmid containing the functional *lacZ::asd2* gene. The double mutant in the chromosomal encoded genes was readily obtained by means of this strategy. The double mutant was denominated SIJUL13 and was demonstrated to contain the replicative plasmid pALASD2.

Inheritance of pALASD2 in the double *asd* mutant

As the double mutant in *asd1* and *asd2* was only obtained in the presence of a complementing *asd*, it was proved whether *asd2* sustented the stable inheritance of pALASD2 in the mutant or not. The double mutant complemented with pALASD2 was cultured in parallel in LB broth and in LB broth supplemented with DAP (100 µg/mL) in the absence of cloramfenicol. The number of CFU was recorded at different times of growth in LB plates supplemented with DAP and scored for the plasmid encoded antibiotic resistance and for growth in unsupplemented LB. In either of the two broth cultures, during the first 12 h, all CFU counted (about 100 each case) were also resistant to the plasmid-encoded marker and able to grow in unsupplemented LB, indicating that plasmid inheritance was being sustained during this time by the plasmidic *asd2*. However at 24 h and after, most of the CFU counted were sensitive to the plasmid encoded marker. The resultant bacteria were able to grow in unsupplemented LB; so, it was reasoned that plasmid loss was not leading to the plasmid-free double mutant but instead that homologous recombination between the plasmid and the chromosome was replacing the *asd2*::kanamycin allele of SIJUL13 with the wild type *asd2* copy previous to plasmid loss. This was corroborated by testing resistance to kanamycin of the resultant colonies. The colonies were sensitive to kanamycin and were demonstrated to contain the wild type *asd2* by Southern blot and PCR. These results suggested again that *Vibrio* cells ignored the presence of DAP in the culture media or gave certain preference to the synthesis of this product, rather than using the exogenous compound.

Colonizing capacity in the infant mouse cholera model

To study a probable effect of each of these genes on the colonization of the small bowel of suckling mouse, it was analyzed the colonizing capabilities of S12CMY12 and 638ASD2K22 in the infant mouse cholera model. Both strains colonized properly the intestine of infant mice; they both showed equivalent viable counts to the parental strain at 24 h of inoculated into the infant mice. Hence, no significant effect on this process could be attributed to mutations in any of these genes in the *in vivo* model (Table 4). Additionally, it was seen that during colonization of the small bowel of mice with 638ASD2K22 (p8LASD2) about one half of the bacteria lost the plasmid. These findings permitted to conclude that functionality of only one of these two genes is sufficient to promote *in vivo* colonization of *Vibrio cholerae* in the intestine of the infant mice.

DISCUSSION

Recently, the complete sequence of the *Vibrio cholerae* genome was annotated and made available to the public.[6] During annotation most of the genes are assigned to a function through sequence comparison. Identification of genes coding for unknown functions and functional testing of those with a predicted

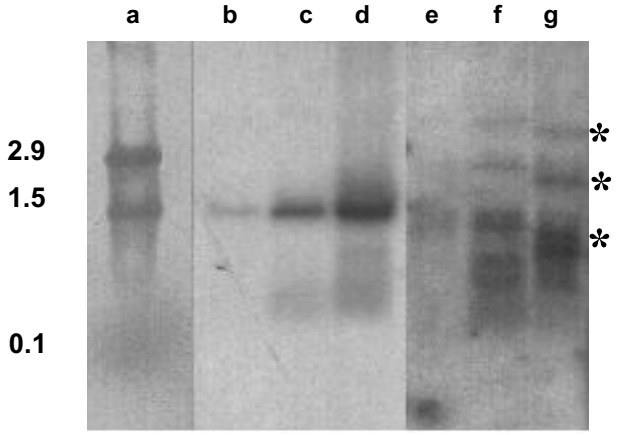

Fig. 6. *Northern blot of total RNAs purified from* V. cholerae *C7258 growing in rich (lanes b and e) and minimal media (a, c, d, and f). Blots were probed with* asd1 *(lanes e and f) or* asd2 *(lanes b, c and d) radiolabeled fragments. Lane a consisted of total RNA from C7258 growing in minimal media; it was stained with methylen blue and taken as a molecular weight marker for the 1.5 and 2.9 kb fragments. Lanes a, b and c contained 10 mg of Total RNA purified from* V. cholerae *C7258, while lanes d, e and f contained 20 mg . Three transcripts of importance, detected with the* asd1 *probe, are indicated with asterisks.*

Table 4. Colonization of *Vibrio cholerae asd* mutants in the small bowel of infant mice 24 h after intragastric inoculation.

Strain	CFU	
	Inoculated[1]	Recovered[2]
638	$5{,}2 \cdot 10^5$	$6{,}0 \cdot 10^5$
S12CMY12	$3{,}3 \cdot 10^5$	$2{,}1 \cdot 10^5$
638ASD2K22	$2{,}1 \cdot 10^5$	$2{,}5 \cdot 10^5$
638ASD2K22/p8LASD2[3]	$4{,}2 \cdot 10^5$	$4{,}2 \cdot 10^5$

[1] Represents the average of the inoculum in three experiments. Independent values ranged between $1 \cdot 10^5$ and $9 \cdot 10^5$. [2] Represents the average of five mice in three independent experiments. Values ranged between $8 \cdot 10^4$ and $9 \cdot 10^5$. [3] One half of the CFU recovered from each mice in LBK were sensitive to ampicillin, indicating plasmid loss. The other half were ampicillin resistant.

function are important tasks of the post-genomic era. In the *Vibrio cholerae* N16961 genome, posted at TIGR (http://www.tigr.org/), VC2036 was annotated as the *asd* gene and VC2107 as a putative *asd*. In this paper we experimentally demonstrate that both genes code Asd functions; being VC2107 the *asd1* gene and VC2036 the *asd2* gene.

Each of the two *asd* genes of *V. cholerae* complements the *asd* defect of *E. coli* χ6097 and each encoded product functions like an ASA dehydrogenase *in vitro*. The predicted polypeptide encoded in each gene shows high level of identity to a different group of Asd proteins found in the Swissprot database; however, no important homology exists between Asd1 and Asd2. This lack of important homology suggests that a duplication process of one of the *asd* genes in *Vibrio cholerae* did not originate the other allele; it is possible interpret that an ancestor *Vibrio cholerae* horizontally acquired a second *asd* gene. This seems plausible since both genes has a G + C content similar to the rest of the genome and a similar codon usage, as posted at the TIGR. Since both genes have coexisted to date, probably the presence of both alleles should have conferred certain adaptive advantage.

It was also demonstrated that *Vibrio cholerae* with one of the two *asd* alleles mutated grows prototrophically at the expense of the other allele. This is a clear evidence that both genes are functional in the bacterium.

Genes *asd1* and *asd2* are located on the same chromosome of *Vibrio cholerae*, namely the replicon I or larger chromosome. The existence of two different genes coding for the same function and placed on the same chromosome is interesting. In bacteria, this generally accounts for the existence of isozymes regulated by different products of the pathway or for isozymes that exert their function at alternative stages. The possibility of considering these two proteins as isozymes regulated by different products of the pathway will need to be addressed in the future. However, with the results presented in this paper, there are more elements to think that each enzyme exerts its function under a different stage. First, prototrophic growth of the *Vibrio cholerae asd2* mutant is suboptimal as judged from the longer doubling time (103 min, Table 3) and long lag period —5 to 7 h— exhibited in minimal media, while the *asd1* mutant grows optimally (doubling time of 46 min) with a normal lag phase —1 h maximally—; second, *asd1* displays lower levels of transcription during laboratory growth than *asd2*; and third, Asd1 and Asd2 require different cofactors for their performance, NAD(H) for Asd1 and NADP(H) for Asd2. Taken together, these results suggest that laboratory growth corresponds to a certain stage or stage II, at which *Vibrio cholerae* lives essentially at the expense of *asd2*, while *asd1* expression is rather low. It merits further analysis studying the conditions leading to the alternative stage or stage I, at which bacterial life occurs more at the expense of *asd1*. It is possible that the transit between these two hypothesized stages during the life cycle of *V. cholerae* has exerted selective pressure to keep active these two genes.

Encouraged by this analysis it was studied the presence of these two genes in other vibrios. In a brief experiment it was analyzed the presence of these two genes in O1 and non-O1 *Vibrio cholerae* by Southern blot. It was observed that N16961 (O1, El Tor, Inaba), C7258 (O1, El Tor, Ogawa), VC12 (O1, El Tor, Inaba), 569B (O1, Classic, Inaba), O395 (O1, El Tor, Ogawa), CA385 (O1, Classic, Ogawa), VC14 (O14), O22 (O22) and SG25-1 (O139), contain a chromosomal copy of each gene in common *Hin*dIII restriction fragments on their chromosomes. The presence of both genes in O1 and non-O1 *Vibrio cholerae* is also indicative that these two genes are needed by this species and have been selected therefore.

As prototrophic growth at the expense of *asd1* in *Vibrio cholerae asd2* mutants is suboptimal, it was investigated whether the single *asd1* or *asd2* mutant was able to colonize efficiently in the infant mouse model of cholera. Both mutants colonized equally well like the parental strain. This probably reflects the existence of enough quantities of methionine, threonine, lysine and isoleucine in the small bowel of infant mice to support optimal replication and colonization at the expense of the unmutated allele. Thus, it was concluded that the colonization process can occur at the expense of either of the two genes.

From this study, it is hypothesized that other bacteria may also have two *asd* genes. In a preliminary search it was not found any description of other bacteria containing two functional *asd* genes. However, *Pseudomonas aeruginosa* is known to have an *asd2* followed by a frame shifted *asd1* beyond the position corresponding to amino acid 114,[7] indicating that they probably coexisted at a given moment. Additionally, it was analyzed the information posted in the Internet for the complete genome sequence of 37 microorganisms (http://www.ncbi.nih.gov/PMGifs/Genomes/mcr.html). Asd belongs to the cluster of orthologous group (COG) 0136. This COG is represented in 24 of 29 bacteria and in 8 of 8 archaea. A single gene was found in each of the archaea —*asd1* type— and in most of the bacteria —*asd1* or *asd2* type—, except in *E. coli*, *P. aeuruginosa*, *V. cholerae*, and *H. influenzae* where COG0136 is represented by two genes. One of these two genes codes for Asd2 like proteins and the other for Asd1 like proteins. The *asd1* like gene was initially denominated *usg1* in *E. coli* and later in *H. inlfuenzae* to indicate their homology. The function encoded in *usg1* has not been well elucidated,[1] but according to the pre-

sent findings it can be related to aspartate semialdehyde dehydrogenase. It has not been studied if the *E. coli* Usg1 has ASA dehydrogenase function when properly expressed, but the fact that *E. coli asd2* mutants are DAP auxotrophs probably indicates that the open reading frame coding for Usg1 is not properly expressed in *E. coli* during laboratory culture.

Both, *asd1* in *V. cholerae* and *usg1* in *E. coli*, are in close association in the chromosome with a *pdxB* homologue to form an operon-like structure. This structural association of *pdxB* and *asd1* is also found in *Shewanella*. However, this is not the case for *H. influenzae* or *P. aeruginosa*, the other bacteria in which two *asd* seemed to exist. Thus, it was concluded, based on the currently available data, that the association *pdxB_asd1* is unusual among bacteria. If this is part of a more general organization it will be revealed after more extensive sequencing of complete genomes. A known cause for the association of two genes in an operon is that both products participate in related processes. The causes for which nature selected for the structural association of *pdxB* and *asd1* in *V. cholerae* and other microbes is not clear, since piridoxal derivatives are cofactors in aminotransfer reactions and the *asd* gene product is a dehydrogenase known to depend on NAD or NADP for functioning in the lysine biosynthetic pathway.

In *Shewanella* sp., it was established the transcriptional activation of its *asd1*-like gene in response to high pressures.[9] As shewanella is a marine organism like *Vibrio cholerae* and both are able to survive and persist for long time in this environment, it might be possible that a pressure inducible *asd* gene is required for inhabitants of this aquatic environment. It was recently described by Bidle and Bartlett that the *Vibrio cholerae* bacteria are able to grow at high pressures[24] without even acquiring a filamentous morphology (Bartlett, personal communication). It would be interesting to know if *asd1* is involved in this survival as it seems to be in *Shewanella* sp.? and if subsistence at high pressures is one of the hypothesized alternative stages at which the *Vibrio* need preferentially *asd1*?

The inability to obtain the double *asd* mutant of *Vibrio cholerae* in the absence of a trans-complementing plasmid-encoded *asd* was not completely understood during the first phase of this research. However, a very likely explanation seemed to be the absence of an efficient mechanism for the entry of DAP into bacterial cells. Several evidences had to be accumulated before reaching this conclusion. The results presented here resembled that of previous authors with other species.[7,25] For example, Cremer *et al*. indicated inefficient mechanisms for DAP entry into *C. glutamicum*[25] and Hoang *et al*., found that *asd* mutants of *P. aeruginosa* needed an exogenous supply of DAP equivalent to 1 mg/mL for growth.[7] The results of this study are similar to theirs in that it was impossible to obtain the double mutant in *Vibrio cholerae* supplying an exogenous source of DAP ranging from 20 to 1 mg/mL . They are also similar in the fact that in the presence of a trans-complementing *asd* functional gene it was readily obtained the double mutant, as well as in that it was not possible to cure the *asd* double mutant from the complementing plasmid (pALASD2) leaving behind the double mutant. It was additionally found that DAP could not substitute for lysine in the TLMI mix added to minimal media to attain the wild type rate of growth in the *asd2* mutant (Table 3), suggesting that vibrio ignored the presence of DAP in the culture media. Although these results pointed to the absence of an efficient mechanism for the entry of DAP in *Vibrio*, the results were not conclusive since Pavelka and Jacobs[26] reported that in *Mycobacterium smegmatis* the apparent inefficiency of DAP entry was related to a high and constitutive expression of *lysA*. LysA is responsible for the conversion of DAP into lysine. Constitutive expression of this protein consumes the DAP incorporated to the cell before it can contribute to the intracellular pool used for the cell wall biosynthesis. This does not seem to be the case with *Vibrio cholerae* since *lysA* transcripts under derepressed conditions are not abundant (in publishing). But to finally address this question, it was investigated whether vibrio could use DAP as a nitrogen source. The wild type strain C7258 was inoculated under three different conditions of growth. The basal medium for culture was SM without the usually added nitrogen source (NH_4Cl, 0.118 %). When DAP (0.5 %) or no supplement of nitrogen source was added, bacterial growth was not observed during the experiment (96 h of incubation at 37 °C). However, when lysine was added, bacterial growth was visible at 24 h and after (Fig. 7). The doubling time of bacteria with lysine (0.5 %) as the sole nitrogen source was 22 h . These results permitted us conclude that *Vibrio cholerae* can grow using lysine but not DAP as an exogenous source of nitrogen. The authors deduce from this that DAP is not efficiently transported into *Vibrio cholerae*.

DAP auxotrophs have been constructed for vaccine purposes in other bacteria. In 1985, Mekalanos *et. al*., indicated the possibility of constructing DAP auxotrophs of *Vibrio cholerae* for vaccine development.[27] The process of constructing DAP auxotrophs in *Vibrio cholerae* for vaccine purposes has consequently been hampered in this study. First, the presence of two functional *asd* genes in the bacterium limited the process and then the fact that *Vibrios*

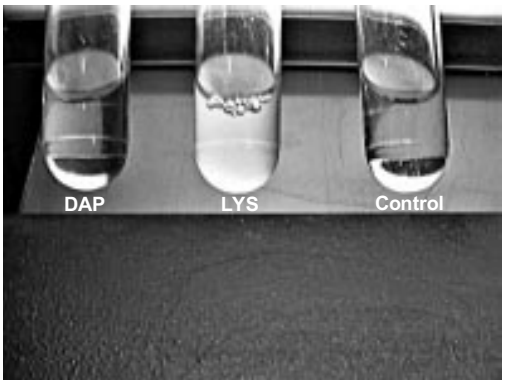

Fig. 7. *Growth of the wild type* Vibrio cholerae *C7258 in the presence of lysine (lys) or DAP as nitrogen source at 37 °C for 96 h. A culture without nitrogen source was photographed as control.*

could not be deprived from their two alleles of this gene complicated the process. This second fact, as discussed previously, indicated an inefficient mechanism for DAP entry into bacterial cells.

Once it was discovered the existence of these two enzymes to exert the same function in *V. cholerae*, it was expected that both enzymes were specific for NADP(H) as cofactor. However, Asd1 was found specific for NAD(H) and Asd2 for NADP(H). It was not found any other description of Asd with NAD(H) requirements in the literature. Instead, the Asd1-like protein of *Amycolatopsis mediterranei* U32[11] was found to be specific for NADP(H). This finding excludes out the possibility that all Asd1 proteins be strictly dependent on NAD(H) and for the moment it should be regarded as a particular feature of *V. cholerae*.

ACKNOWLEDGEMENTS

We are grateful to Roy Curtiss III for providing us with *E. coli* χ6097, to R.A. Finkelstein for providing *Vibrio cholerae* C7258 and to Christa Baluja and Chryslaine Rodríguez for performing the synthesis of ASA. Arlenys Moreno assisted in the conservation and maintenance of laboratory stocks of bacterial strains. We acknowledge Jorge Benítez for the support and trusting given to the embryo of this idea.

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