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A Short Chain NAD(H)-Dependent Alcohol Dehydrogenase (HpSCADH) from Helicobacter Pylori: a Role in Growth under Neutral and Acidic Conditions.

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A short chain NAD(H)-dependent alcohol dehydrogenase (HpSCADH) from *Helicobacter pylori:* A role in growth under neutral and acidic conditions

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ABSTRACT

Toxic aldehydes produced by alcohol dehydrogenases have been implicated in the pathogenesis of *Helicobacter pylori*-related damage to the gastric mucosa. Despite this, the enzymes that might be responsible for producing such aldehydes have not been fully described. It was, therefore, of considerable interest to characterize the alcohol oxidizing enzymes in this pathogen. Previous work in this laboratory characterized two such *H. pylori* enzymes that had broad specificity for a range of aromatic alcohol substrates. An enzyme with broad specificity for aliphatic alcohols is likely to be required in order that *H. pylori* can metabolize the wide range of substrates encountered in the gastric mucosa. In this study we describe HpSCADH, an alcohol dehydrogenase from *H. pylori* 26695 with broad specificity for aliphatic alcohols. HpSCADH was classified in the cD1e subfamily of classical short chain alcohol dehydrogenases. The enzyme was a monomer of approximately 29 kDa with a preference for NAD+ as cofactor. Pyrazole was found to be a competitive inhibitor of HpSCADH.

The physiological role of this enzyme was explored by construction of an HpSCADH isogenic mutant. At pH 7.0 the mutant showed reduced growth which became more pronounced when the pH was lowered to 5.0. When pyrazole was added to wild type *H. pylori* cells it caused growth profiles to be reduced to those of the isogenic mutant suggesting that HpSCADH inhibition alone was responsible for growth reduction. Taken together, the data relating to the alcohol metabolizing enzymes of this pathogen indicate that they play an important role in *H. pylori* growth and adaptation to acidic environments. The therapeutic potential of targeting *H. pylori* alcohol dehydrogenases is discussed.

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1. Introduction

Helicobacter pylori is implicated in the pathogenesis of gastric lymphoid tissue-associated B-cell lymphoma (Von Herbay et al., 1995) and gastric adenocarcinoma in humans (Parsonnet et al., 1991). Furthermore, 90–95% of duodenal ulcers in Europe originate from *H. pylori* infection (Krah et al., 2004).

A role for aldehyde toxicity in the pathogenicity of *H. pylori*-mediated gastric disease has been proposed. Specifically, it was suggested that alcohol dehydrogenases of *H. pylori* contribute its pathogenicity by oxidizing alcohols to produce toxic aldehydes: the aldehydes then react with and modify proteins in the gastric mucosa to cause inflammation. This inflammatory process may lead to gastric adenocarcinoma in some cases (Salmela et al., 1997; Homann et al., 1997; Salaspuro, 2003; Matysiak-Budnik et al., 1995;

Figura, 1997). Ethanol-derived acetaldehyde has been the focus of much of the concern for gastric carcinoma (Salaspuro, 2011).

This laboratory has undertaken a systematic study of the pyridine linked alcohol oxidizing enzymes of *H. pylori*. The annotated genome of *H. pylori* 26695 (Tomb et al., 1997) identifies an aldo-keto reductase (HpAKR), a cinnamyl alcohol dehydrogenase (HpCAD) and a putative short chain alcohol dehydrogenase gene (HpSCADH). These pyridine linked oxidoreductases might all be expected to play a role in alcohol oxidation. Two of these enzymes (HpCAD and HpAKR) have been extensively studied in this laboratory (Mee et al., 2005; Cornally et al., 2008). Both HpCAD and HpAKR showed broad substrate specificity for aromatic alcohols. Interestingly, HpAKR was shown to play an important role in the growth of *H. pylori* under acidic conditions and is thought to be involved in the adaptation of this bacterium to growth in the gastric mucosa (Cornally et al., 2008). An enzyme with specificity for aliphatic alcohols is presumably necessary to allow this bacterium to catalyze the oxidation of a wider range of physiological substrates.

This study identifies an enzyme capable of oxidizing aliphatic alcohols which provides the organism with the ability to

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metabolize a broad range of aliphatic and aromatic substrates. The physiological role of this dehydrogenase was of considerable interest since it appeared to be the dominant enzyme in the metabolism of non-aromatic alcohols in the organism. To explore this issue further an isogenic HpSCADH-negative mutant of *H. pylori*, was constructed using insertional mutagenesis. The mutant showed reduced growth compared to the wild type at both neutral and acidic pH indicating a role for this dehydrogenase in adaptation to the gastric mucosa. We identify pyrazole as an inhibitor of this enzyme and show that this compound can reduce the growth of *H. pylori* in culture.

2. Materials and methods

2.1. Cloning of the HpSCADH gene

All DNA manipulations were performed as described previously (Sambrook and Maniatis, 1989). Oligonucleotides used for PCR amplification of the HpSCADH gene, using *H. pylori* 26695 genomic DNA as template, were designed using the putative short chain alcohol dehydrogenase gene sequence from *H. pylori* 26695, available at the NCBI database (NP_207155.1). The forward (5′-CGCCATATGGCGCACATT-3′) and the reverse (5′-CGCGGATCCAGGGTTTTT ATG GGTG-3′) primers were designed to introduce *Nde* I and *BamH* I restriction enzyme cleavage sites at the N-terminus and C-terminus, respectively. Polymerase Chain Reactions were performed using a Perkin-Elmer 2400 thermal cycler with *Taq* DNA polymerase (Boerhinger Mannheim, Germany). Primers were obtained from Sigma-Genosys (UK). For standard amplifications approximately 100 ng of template DNA was mixed with 5 pmol of each specific primer in a reaction volume of 25 μl. Reactions were performed by denaturing DNA at 94 °C for 2 min 15 s, annealing at 45 °C for 30 s and extension at 72 °C for 45 s. A total of 30 cycles were performed. The amplified PCR product was cloned into pET16(b) (Novagen). The resulting construct was named pET-HpSCADH. The positive clones were confirmed by DNA sequencing (Eurofins MWG, Ebensburg, Germany).

2.2. Purification of the HpSCADH gene product

Escherichia coli BL21 (DE3) plysS transformed with the pET16(b)-HpSCADH construct were grown at 37 °C in LB medium containing 50 μg/ml ampicillin. At an OD_{600} of 0.8 the expression of HpSCADH was induced by the addition of 1.0 mM IPTG at 25 °C and incubated for 14 h with constant shaking (280 rpm). Cells were harvested and resuspended in lysis buffer (20 mM Tris–HCl, pH 7.9, 5 mM imidazole, 200 mM NaCl) and lysed by sonication for 130 s on ice (VC-750 ultrasound generator, Sonics and Materials Inc.). Cell debris was collected by centrifugation and the supernatant was applied to a nickel-charged iminodiacetic acid column (Novagen) equilibrated with lysis buffer. The Ni^+ column was washed with 2 l of wash buffer, which was the same as the lysis buffer before elution with 20 mM Tris–HCl (pH 7.9) containing 200 mM NaCl and 300 mM imidazole. Fractions containing purified recombinant HpSCADH were identified by SDS-polyacrylamide gel electrophoresis. Positive fractions were pooled and dialyzed against 2 l of pre-chilled 0.2 M potassium phosphate, pH 7.4, for 36 h with three changes.

2.3. Enzyme assay

Kinetic parameters were determined spectrophotometrically at 37 °C using an Agilent 8453 diode array spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). The purified HpSCADH was assayed for both the oxidation of alcohols (forward reaction) and the reduction of aldehydes (reverse reaction). Activities with alcohols were measured in a final volume of 2.0 ml in 0.2 M potassium phosphate buffer (pH 7.5) containing 0.2 mM NAD^+ and alcohol substrate, typically 50 mM for ethanol. The formation of NADH at 340 nm was followed. The molar extinction coefficient (ε) used (pH 7.5) was $\varepsilon_{340} = 6220\,M^{-1}\,cm^{-1}$ for NADH. The oxidation of cinnamyl alcohol or reduction of cinnamaldehyde was monitored at 366 nm (Larroy et al., 2003). Steady-state parameters were determined by fitting initial rates to the Michaelis–Menten equation using ENZ-FITTER (Elsevier Biosoft, Cambridge, UK).

Activities towards different aldehydes were assayed in a 2 ml reaction mixture containing 0.2 M potassium phosphate buffer (pH 7.5) with 0.2 mM NADH and varying aldehyde concentrations. The decrease in NADH absorbance at 340 nm was followed to measure enzyme activity.

2.4. Effect of pH and temperature on HpSCADH activity

The optimum pH for HpSCADH activity was determined in a 2 ml assay mixture containing 0.2 mM $NADH/NAD^+$ and 1 mM propionaldehyde/50 mM 1-propanol as substrates for the reduction and oxidation half reactions respectively, in a standard assay mixture at 37 °C. Different pH values were generated using the following buffers: pH 4–5: 50 mM sodium citrate, pH 6–8: 50 mM potassium phosphate and pH 9–10: 50 mM glycine.

To examine the effect of temperature, HpSCADH was assayed in the temperature range 20–60 °C in a 2 ml assay mixture containing 0.2 mM NAD^+, 50 mM of 1-propanol as substrate and ∼11 μg of HpSCADH ml^{-1} of assay mixture. The activity at various temperatures was studied by incubating the enzyme in 0.2 M potassium phosphate buffer (pH 7.5) at different temperatures for 30 min. Each sample was then assayed for residual activity.

2.5. Bacterial strains and plasmids

H. pylori strains 26695 and 1061 were originally obtained from A. Van Vliet and J. Kusters (Erasmus MC University Medical Centre, Rotterdam, The Netherlands). The wild type strains were maintained on Columbia Blood Agar plates containing 5% (v/v) defibrinated horse blood while the medium for the HpSCADH mutant was supplemented with kanamycin (20 μg/ml) for selection. Plates were incubated in a microaerophilic-humidified atmosphere generated using a MART Anoxomat system (Lichtenvoorde, the Netherlands). For liquid culture, strains were grown in Brucella Broth (Sigma) supplemented with 7% (v/v) foetal calf serum. Cultures were grown in 25 cm^2 cell culture flasks with constant shaking (120 rpm) at 37 °C in an orbital incubator shaker.

An acidic environment was created using Brucella broth which was adjusted to the desired pH using 0.1 M HCl (after the addition of foetal calf serum) and subsequently filter sterilized. *E. coli* was grown in LB broth and on LB agar, as required. The antibiotics used for selection purposes were ampicillin (50 μg/ml) and kanamycin (20 μg/ml).

2.6. DNA manipulations

All DNA manipulations were carried out according to standard procedures (Sambrook and Maniatis, 1989). Transformation of *E. coli* cloning host (DH5α; Invitrogen) was performed according to the manufacturer's protocol. Natural transformation of *H. pylori* with plasmid constructs was carried out as described in Smeets et al. (2000). All oligonucleotide primers were obtained from Eurofins MWG (Ebensburg, Germany).

2.7. Construction of HpSCADH isogenic mutant

The HpSCADH gene was inactivated in *H. pylori* strain 1061 by gene disruption. The purified PCR-amplified *Hp0357* (HpSCADH
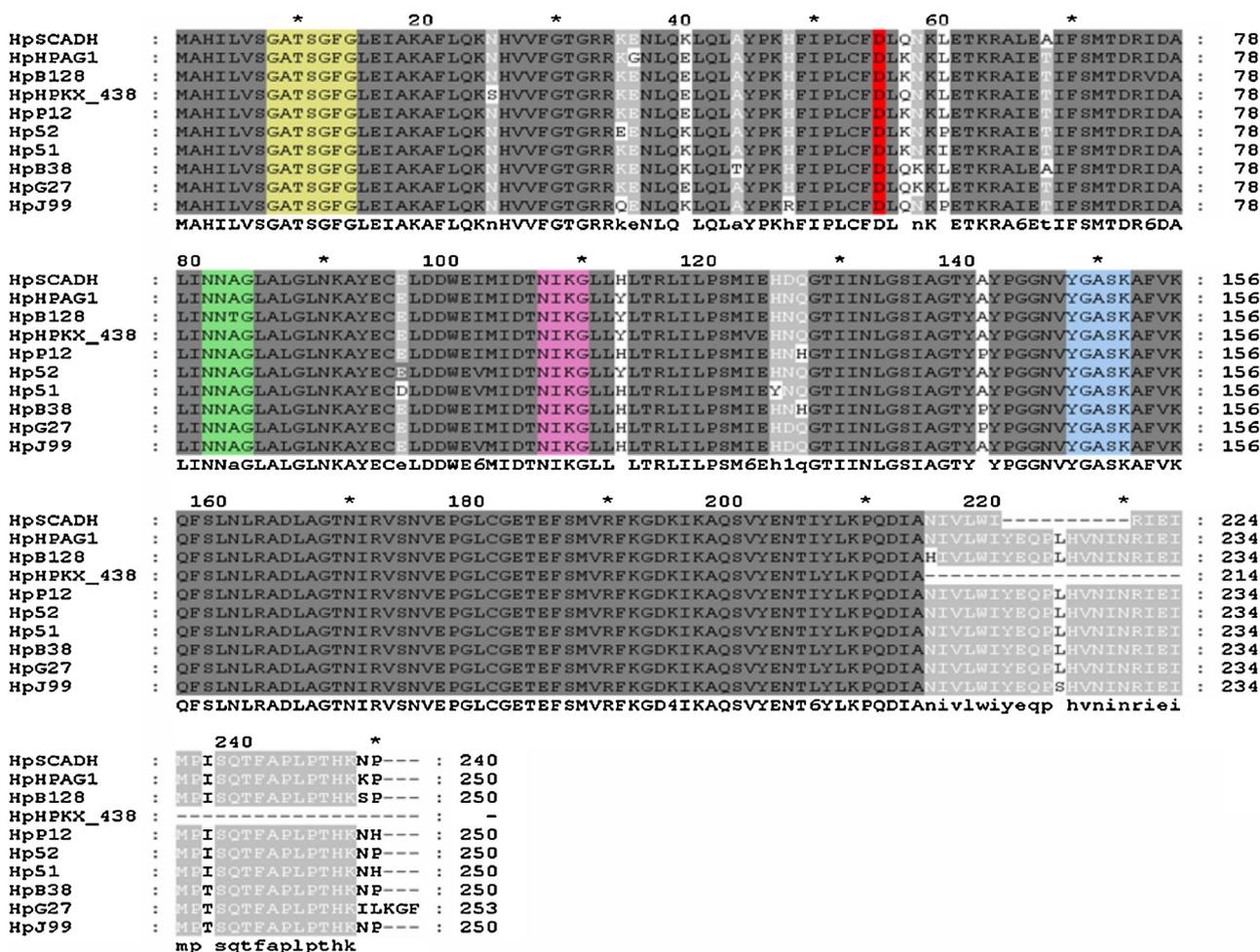
Fig. 1. Sequence alignment of short chain alcohol dehydrogenases from different strains of *H. pylori*. Clustal W alignment of HpSCADH from *H. pylori* 26695 against short chain alcohol dehydrogenase/reductases (SDR) from various strains of *H. pylori*. Sequence similarity is indicated by (dark gray) exact matches, (light gray) strong similarity, (no shading) weak similarity. Conserved motifs typical of classical SDRs such as N-terminal cofactor binding site (yellow), active site (blue), a conserved part of the active site (pink), aspartic acid at position 55 (red) and central β-sheet stabilizing motif (green) were present in HpSCADH as well as in the SDRs from various other strains of *H. pylori*. **Q4** Clustal W sequence alignment was imported to Genedoc (http://www.psc.edu) for manipulation of the aligned sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gene) was ligated into the cloning vector pGEM-T Easy (Promega) by TA cloning and transformed into *E. coli* DH5α. The primers used to amplify *Hp0357* were: 5′-ATGGCGCACATTTTAGTTAGCGG-3′ as the forward primer and 5′-AGGGTTTTTATGGGTGGGTAG-3′ as the reverse primer. A 1.5-kb PCR product from plasmid pJMK30 containing a gene encoding resistance to kanamycin was amplified using 5′-TGATCAGTAAAACGACGGCCAGT-3′ as forward primer and 5′-TGATCAAACAGCTATGACCATG-3′ as reverse primer. The PCR amplified kanamycin cassette (aphA-3) containing the unique *Bcl* I site at both ends was subcloned into pGEM-T Easy vector (Promega) by TA cloning and transformed into *E. coli* DH5α (Invitrogen). Plasmid DNA isolated from *dam+ E. coli* (*e.g.* DH5α) is completely resistant to cleavage by *Bcl* I, which cleaves at GATC sites. These sites are blocked by *dam* methylation (methylation at the N^6 position of the adenine in the sequence 5′-GATC-3′). This was circumvented by transforming the pGEM:HpSCADH and pGEM:aphA-3 constructs into a *dam−/dcm−* strain of *E. coli* (New England Biolabs, Herts, England).

Both pGEM:Hp0357 and pGEM:aphA-3 (isolated from *dam−/dcm− E. coli*) were digested with *Bcl* I to generate cohesive ends. The *Bcl* I digested kanamycin cassette was cloned into the unique *Bcl* I site within the coding region of HpSCADH gene yielding pGEM:HpSCADH::aphA-3 construct (Fig. 5). Disruption of the HpSCADH gene by the kanamycin cassette was confirmed by DNA sequencing (Eurofins MWG, Ebensburg, Germany). The mutant construct was used for natural transformation of *H. pylori* 1061. *H. pylori* genomic DNA was purified using the Wizard® Genomic DNA Purification Kit (Promega, USA).

3. Results

3.1. Sequence analysis/classification

A protein BLAST analysis of HpSCADH revealed the highest sequence similarity with a number of putative short chain alcohol dehydrogenases and oxidoreductases from pathogens belonging to various bacterial families such as *Actinobacillus pleuropneumoniae* (ZP_00134882.2, 63% identity), *Aggregatibacter actinomycetemcomitans* (YP_003255986.1, 63% identity), *Mannheimia haemolytica* (ZP_05988835.1, 61% identity), *Aeromonas hydrophila* (YP_855033.1, 60% identity), *Haemophilus influenzae* (ZP_04466822.1, 58% identity) and *Streptococcus mutans* NN2025 (YP_003484911.1, 57% identity).

Clustal W sequence alignment of HpSCADHs across various strains of *H. pylori* shows several conserved motifs (Fig. 1). These motifs include an N-terminal cofactor binding site (GxxxGxG, yellow), the active site motif (YxxxK, blue) and a mid-region conserved site (NNAG, green) (Jornvall et al., 1999; Van der Oost et al., 2001;
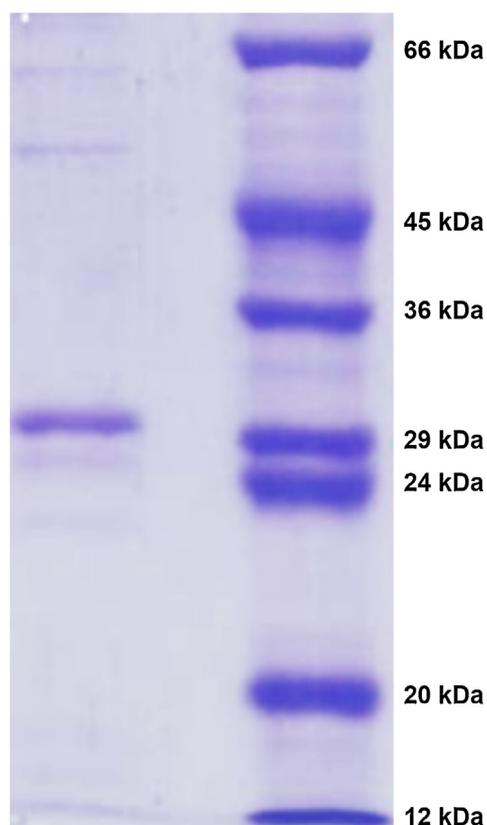
Fig. 2. SDS-PAGE of purified HpSCADH. (Lane 1) from left: purified HpSCADH protein with an approximate molecular mass of 29 kDa. (Lane 2) protein standard marker proteins with molecular weight marked on right.

Fig. 3. Characterization of HPSCADH: (A) HpSCADH activity as a function of pH for the oxidation (red) and reduction (black) half reactions. The buffers used were as follows: pH 4–5: 0.2 M sodium citrate, pH 6–8: 0.2 M potassium phosphate and pH 9–10: 0.2 M glycine. Assays were carried out at 37 °C as described in Section 2. The assay mixture for the oxidation reaction contained 50 mM 1-propanol while for reduction 1 mM propionaldehyde was used as substrate. (B) Temperature dependence of HpSCADH activity. Initial rates of HpSCADH mediated oxidation of ethanol (50 mM) over a range of temperatures. Activity was measured using standard conditions, as outlined in Section 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Kallberg et al., 2002). Based on these observations HpSCADH can be classified as a classical short chain alcohol dehydrogenase. An additional conserved motif (NhxG, pink, Fig. 1), which is involved in positioning the active site lysine residue and forming part of a postulated proton relay (Filling et al., 2002), is typical of classical SCADHs as is the presence of an aspartic acid at position 55 as part of the cofactor binding site (Fig. 1, red). The size of HpSCADH (250 amino acid residues) is also characteristic of classical SCADHs (Kallberg et al., 2002).

NADH-preferring classical SDRs have an acidic residue at the C-terminal of the 2nd beta-strand (key position 36) (Wierenga et al., 1985, 1986) unlike NADPH binding enzymes that have two basic residues (Tanaka et al., 1996). The HpSCADH sequence contains a glutamic acid at position 36 (Fig. 1) indicating that it is expected to show a preference for NADH over NADPH. Hence, HpSCADH belongs to the cD1e subfamily of the classical NAD(H)-preferring SDRs.

3.2. Expression and purification of HpSCADH

The recombinant HpSCADH was expressed with an N-terminal His-tag. Following purification and dialysis HpSCADH was stored in 0.2 M phosphate buffer (pH 7.5) at −20 °C. No loss of enzyme activity was observed when stored under these conditions for more than 1 month. Typically, 1 l of culture yielded ~5 mg of purified HpSCADH. The HpSCADH eluted from the Ni-column was ~95% pure as shown by 12% SDS-polyacrylamide electrophoresis gels with a relative molecular mass of ~29 kDa (Fig. 2). Control activity measurements with E. coli BL21 (DE3) plysS cell extracts lacking the HpSCADH construct showed no short chain alcohol dehydrogenase activity.
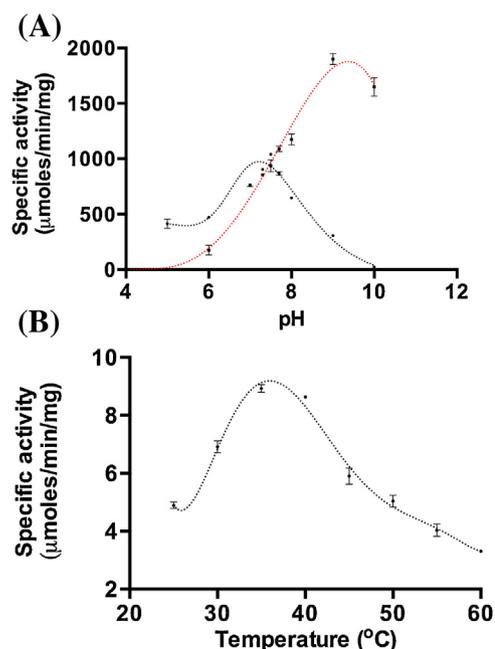
3.3. Characterization: optimum pH and temperature

The activity of HpSCADH was dependent on pH for both the reduction and oxidation half reactions (Fig. 3A). In the reduction reaction there was a narrow peak of maximum activity at approximately pH 7.5. The oxidation reaction showed less marked dependence in the pH range of 7–9 with a broad peak centred around pH 9 (Fig. 3A).

The effect of temperature on HpSCADH activity is shown in Fig. 3B. The reaction rate increases up to about 38 °C and then decreases rapidly. The optimum range was 35–40 °C. All experiments were carried out in triplicate.

3.4. Substrate specificity

HpSCADH showed no activity with NADP(H). Its activity was tested with a range of aliphatic and aromatic alcohol/aldehydes, ketones and dicarbonyls (Tables 1A and 1B). Steady-state parameters were determined for the most active substrates (Table 2). All assays were carried out at pH 7.5, and at 37 °C.

Greatest catalytic efficiency with respect to the alcohol substrates tested was seen with 1-propanol which had a k_{cat}/k_m value of 118.4 s^{-1} mM^{-1}. The enzyme showed no activity with aromatic alcohols except for a discrete activity with cinnamyl alcohol. Of the aldehydes evaluated as substrates, butyraldehyde and cinnamaldehyde yielded k_{cat}/k_m values of 29,198 s^{-1} mM^{-1} and 540 s^{-1} mM^{-1} respectively. Some activity was seen with pyridine aldehydes.

The effect of branched chain alcohol substrates on HpSCADH activity was explored by comparing primary, secondary and tertiary butanol. It was found that HpSCADH catalyzed the oxidation of 1-butanol at a rate that was 1.5-fold greater than 2-butanol

Table 1A
Substrate screening of the *H. pylori* HpSCADH catalyzed oxidation of alcohols.

Substrate	Substrate concentration (mM)	Enzyme activity (μmol/min/mg)
Aliphatic alcohols		
Methanol	50	NDA
Ethanol	50	63.2
1-Propanol	50	625.5
2-Propanol	50	110.4
1,2-Propandiol	50	NDA
1-Butanol	50	79.5
2-Butanol	50	54.1
Tert-butanol	50	NDA
2-Methyl-1-butanol	50	NDA
1-Pentanol	50	270
2-Pentanol	10	NDA
4-Penten-1-ol	10	NDA
Hexanol	50	146.9
Octanol	10	NDA
Aromatic alcohols		
Benzyl alcohol	10	NDA
4-Methoxybenzylalcohol	10	NDA
4-Nitrobenzyl alcohol	10	NDA
Cyclohexanol	50	NDA
Cinnamylalcohol	10	865.1
Dicarbonyls		
Phenylglyoxal	1	94.2
Methylglyoxal	1	155.4

0.2 mM of NAD$^+$ was used in all assay mixture. The dicarbonyl substrates were dissolved in 100% methoxyethanol. All alcohol substrates were dissolved in 0.2 M phosphate buffer (pH 7.5) except hexanol and octanol which were dissolved in 100% methoxyethanol. NDA is no detectable activity.

while no detectable activity was observed with tertiary butanol. Thus, it was clear that the degree of branching strongly influenced catalysis with straight chain aliphatic substrates being optimal. Other aromatic alcohols, aldehydes and ketones showed no detectable activity (Tables 1A and 1B). Among the dicarbonyls tested HpSCADH showed higher activity towards methylglyoxal than phenylglyoxal.

Table 1B
Substrate screening of the *H. pylori* HpSCADH catalyzed reduction of aldehydes.

Substrate	Substrate concentration (mM)	Enzyme activity (μmol/min/mg)
Aliphatic aldehydes		
Formaldehyde	1	NDA
Acetaldehyde	1	272.9
Butyraldehyde	0.1	575.6
2-Methylbutyraldehyde	1	NDA
Propionaldehyde	0.1	556.9
Valeraldehyde	1	277.9
Hexanal	1	371.5
Octanal	1	1342.2
Crotanaldehyde	1	834.5
Glyceraldehyde	1	NDA
Aromatic aldehydes		
Cinnamaldehyde	0.5	509.8
Pyridine 2 aldehyde	1	688.8
Pyridine 4 aldehyde	1	135.6
Benzaldehyde	1	NDA
Ketones		
Acetone	50	NDA
Propanone	50	NDA
Butanone	50	NDA
Pentanone	50	NDA
Acetophenone	1	NDA
2,2,2-Trifluroacetophenone	1	NDA
Cyclohexanone	10	NDA

0.2 mM of NADH was used in all assay mixture. All aldehyde and ketone substrates were dissolved in 100% methoxyethanol. NDA is no detectable activity. Methoxyethanol was not a substrate under these conditions.

Table 2
HpSCADH substrate specificity. Kinetic parameters of HpSCADH from *H. pylori*. All data are mean of triplicate measurements. Assays were carried out as described in Section 2.

Substrate	k_m (mM)	k_{cat} (s^{-1})	k_{cat}/k_m (s^{-1} mM^{-1})
NADH[a]	0.026 ± 0.0043	362.2 ± 15.4	13932.6
NAD[a]	0.036 ± 0.007	510.8 ± 26.6	14187.5
Acetaldehyde	1.2 ± 0.61	44.0 ± 11.7	52.8
Butyraldehyde	0.0036 ± 0.0003	105.1 ± 1.5	29198.0
Propionaldehyde	0.18 ± 0.002	86.9 ± 1.9	482.7
Valeraldehyde	1.07 ± 0.39	47.9 ± 9.4	44.8
Hexanal	0.99 ± 0.11	63.8 ± 5.6	64.4
Cinnamaldehyde	0.3 ± .06	162.0 ± 14.9	540.0
Ethanol	7.2 ± 0.67	3.0 ± 0.1	0.4
1-Butanol	3.1 ± 0.30	2.7 ± 0.1	0.9
1-Propanol	1.8 ± 0.25	213.1 ± 5.6	118.4
2-Propanol	280.93 ± 73.85	310.1 ± 43.5	1.1
1-Pentanol	12.9 ± 1.11	104.8 ± 2.4	8.1

[a] Substrate used for the determination of the kinetic constants of NADH and NAD$^+$ was 1 mM butyraldehyde and 50 mM ethanol respectively.

Table 3
Effectors of HpSCADH activity. **Q5**

Compound	Concentration (mM)	Relative activity (%)
Pyrazole	0.1	0
Sodium valproate	1	46
Chloral hydrate	1	100
2-Mercaptoethanol	1	100
EDTA	1	78
ZnSO$_4$[b]	1	70
MgCl$_2$	1	100
KCl	1	90

[a] HpSCADH mediated oxidation of 1 mM 1-butanol in a standard assay mixture was assumed to be the control at 100%. The activity in the presence of various compound are expressed as the percentage of 1-butanol oxidase activity.

[b] Initial stock solutions of these compound were prepared in DMSO. All assays were carried out as described in Section 2. Data are mean of triplicate measurements.

3.5. Inhibition studies

The influence of various effector molecules on HpSCADH activity is summarized in Table 3. The presence of Mg^{2+} and K$^+$ had no significant effect on HpSCADH activity while ZnSO$_4$[1] showed mild inhibition. EDTA at 1 mM concentration reduced the enzyme activity by ~22%. Sodium valproate, a potent inhibitor of manyaldo-keto reductases (Hinshelwood et al., 2002; Kuhn et al., 1995; Todaka et al., 2000), was found to inhibit HpSCADH activity by 55% at a concentration of 1 mM with little further inhibition seen at elevated concentrations. Chloral hydrate showed no inhibition. Treatment with 2-mercaptoethanol had no effect on the enzyme.

Pyrazole is a known potent inhibitor of many alcohol dehydrogenases with K_i values typically in the low micromolar region (Goldberg and Rydberg, 1969; Li and Theorell, 1969; Reynier, 1969). HpSCADH activity was found to be fully inhibited by 100 μM pyrazole (Table 3). Inhibition was of a competitive-type with respect to 1-butanol (Fig. 4) and a K_i of 10.05 ± 0.03 μM was estimated.

3.6. Disruption of HpSCADH by insertional mutagenesis and characterization of the isogenic mutant

HpSCADH was markedly specific for aliphatic alcohols which made it interesting to evaluate its role in alcohol and aldehyde

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[1] Initial stock solutions of these compounds were prepared in DMSO. All assays were carried out as described in Section 2. Data are mean of triplicate measurements.
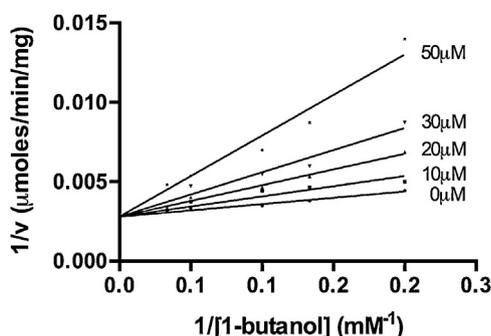
Fig. 4. Kinetics of pyrazole inhibition. Competitive inhibition plot for estimation of K_i ($10.05 \pm 0.03\,\mu M$) for pyrazole. Plot of reciprocal of rate $1/v$ versus $1/1$-butanol concentration at increasing inhibitor concentrations (pyrazole). The concentrations of pyrazole employed were 0, 10, 20, 30 and 50 μM. Data points for individual lines were fitted to the Michaelis–Menten equation to estimate k_m (inset). The plot of the apparent k_m values against the pyrazole concentration was linear.

metabolism in the organism. This was explored by generating an isogenic mutant of HpSCADH. The mutant was generated by insertion of a kanamycin antibiotic resistance cassette within the coding region of the HpSCADH gene (Fig. 5).

H. pylori 1061 (wild type) and HpSCADH mutant strains were grown under standard microaerobic conditions in Brucella broth medium supplemented with FCS (7%, v/v) at pH 7.0. The optical density of the liquid cultures was monitored at 600 nm every 6 h over a period of 48 h (Fig. 6A). It is evident from the growth profiles that the HpSCADH mutant was compromised compared to the parental strain at pH 7.0 suggesting a role for HpSCADH in the metabolism/growth of *H. pylori*. The growth of the isogenic mutant was even further compromised under acidic conditions (Fig. 6B). This finding was of particular interest since a previous report from this laboratory showed that *H. pylori* (HpAKR) played a role in acid adaptation (Cornally et al., 2008) it seems that the HpSCADH is somewhat similar. Growth characteristics at a pH 6.0 for both the wild type and HpSCADH mutant strains were similar to those seen at pH 7.0 (data not shown). The growth rate of both the wild type and the mutant were compromised at pH 5.0 (Fig. 6B). The growth rate of the HpSCADH mutant was severely compromised beyond 8 h of growth, compared to the wild type.

3.7. Effect of pyrazole on the growth of wild type and HpSCADH negative mutant of H. pylori 1061

In this study we have shown that pyrazole is an inhibitor of HpSCADH. It has also been shown that knockout of this enzyme causes growth inhibition of *H. pylori* (Fig. 6A). It was, therefore, of interest to assess the growth of this mutant *H. pylori* compared to the wild type, in the presence and absence of pyrazole.

The wild type *H. pylori* showed impaired growth in the presence of 20 μM pyrazole similar to the HpSCADH mutant without pyrazole (Fig. 7). The growth of the HpSCADH mutant *H. pylori* was unaffected by the presence of the same concentration of pyrazole up to 22 h (Fig. 7). However, the mutant did show reduced growth after 22 h.

4. Discussion

4.1. Sequence analysis

HpSCADH showed high sequence identity to other putative short chain alcohol dehydrogenases from various bacterial pathogens. Its preference for NAD(H) over NADP(H) serves to group HpSCADH with other NAD(H) preferring SDRs such as 3α,20β-hydroxysteroid dehydrogenase, 7α-hydroxysteroid
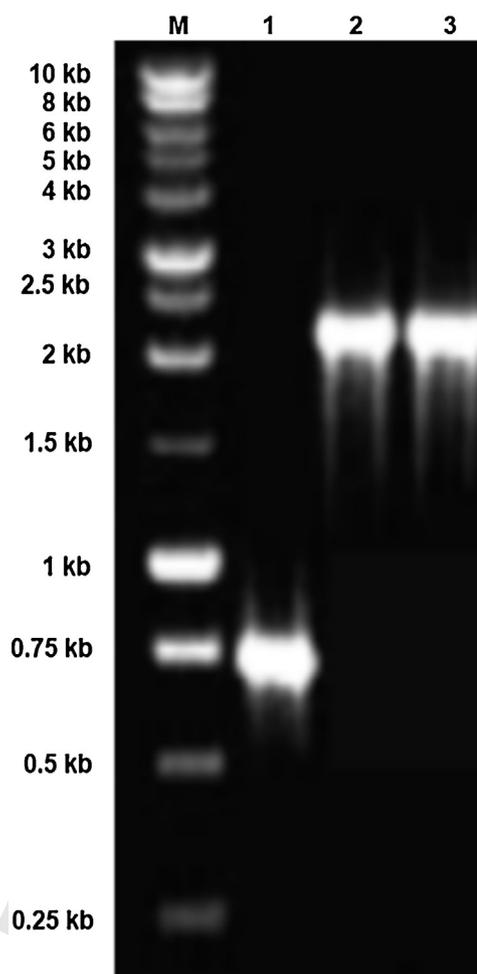

Fig. 5. Integration of the aphA-3 disrupted HpSCADH into the genome of *H. pylori* 1061. 0.8% agarose gel indicating the presence of the inserted aphA-3 cassette in the HpSCADH negative mutant of *H. pylori* 1061. Lanes 2 and 3 show a 1.5 kb increase in size of the HpSCADH gene (chromosomal) due to the presence of the inserted aphA-3 cassette amplified from *H. pylori* 1061. Lane 1 contains the HpSCADH gene, with an approximate size of 750 bp, amplified from the parental wild type *H. pylori* 1061. Lane M contains the 1 kb DNA ladder (Promega). PCR was carried out as described in Section 2.

dehydrogenase, 2,3-dihydroxybiphenyl dehydrogenase, 2,3-butanediol dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase type 2 and dihydropteridine reductase that also have an acidic residue at position 36. This acidic residue interacts with the 2′- and 3′-hydroxyl groups of the adenine ribose moiety in enzymes that are NAD(H)-binding (Wierenga et al., 1985).

Sequence alignment studies with the sequence of highest identity showed that most of the conserved motifs of SDRs are found in the HpSCADH gene sequence.

4.2. Substrate specificity

Short-chain alcohol dehydrogenases/reductases (SDR) are a large superfamily of proteins with a large diversity of functions such as lipid, carbohydrate, amino acid or xenobiotic metabolism. HpSCADH has a clear preference for aliphatic alcohols. k_m values were generally within 0.1–2 mM for aldehyde substrates and 1–15 mM for alcohol substrates with the exception of 2-propanol, for which a higher k_m was obtained (280 mM). HpSCADH showed no detectable activity towards range of aromatic alcohols aldehydes, dicarbonyls and ketones tested in this study. A discrete activity with cinnamaldehyde was observed for HpSCADH. This is
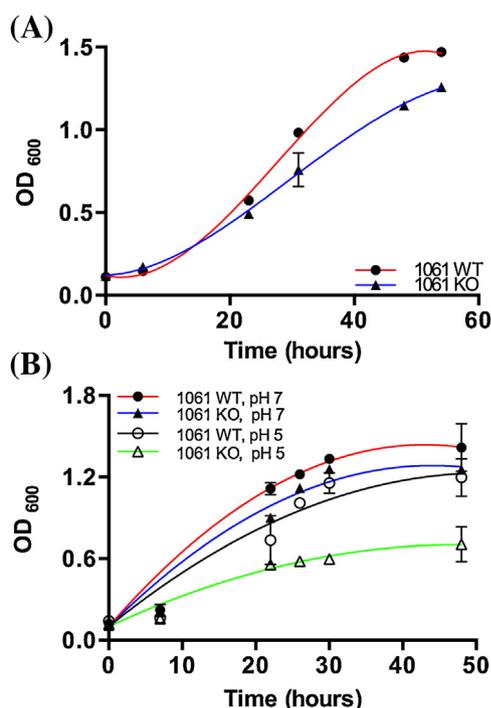
Fig. 6. Growth profiles of the isogenic HpSCADH negative mutants of *H. pylori* at neutral and acidic conditions. (A) The growth characteristics of both *H. pylori* 1061 wild type (red) and HpSCADH knockout mutant (blue) at pH 7.0 in Brucella broth supplemented with 7% foetal calf serum and kanamycin (20 μg/ml). (B) The growth characteristics of both *H. pylori* 1061 wild type and HpSCADH mutant in Brucella broth supplemented with 7% foetal calf serum and kanamycin (20 μg/ml) at pH 7.0 (control) and pH 5.0. All results are the mean ± the SEM (*n* = 4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

probably due to the fact that the aldehyde group in cinnamaldehyde is more accessible, due to its location relative to the aromatic moiety compared to the other aromatic substrates tested in this study. Unlike CAD from *H. pylori*, HpSCADH showed no measurable aldehyde dismutation activity with cinnamaldehyde (data not shown).

These substrate specificity studies point to a separation of roles for enzymes involved in alcohol metabolism in *H. pylori* with HpCAD and HpAKR oxidizing mainly aromatic alcohols and HpSCADH mainly aliphatic alcohols. Combined they provide the organism with the ability to oxidize a wide range of alcohol substrates.
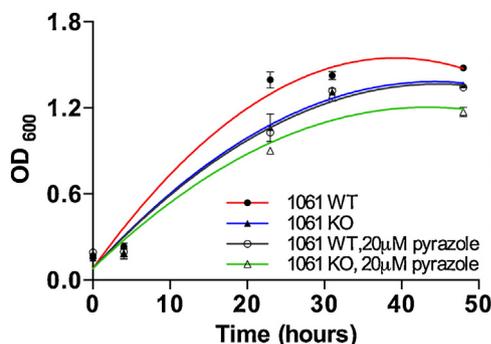

Fig. 7. Effect of pyrazole on the growth of *H. pylori* 1061 wild type and HpSCADH mutant at pH 7.0. Growth of *H. pylori* 1061 wild type and HpSCADH negative mutants in Brucella broth supplemented with 7% foetal calf serum and kanamycin (20 μg/ml) with and without pyrazole (20 μM). Results are the mean ± the SEM.

The substrate preference for HpSCADH overlaps somewhat with HpCAD which, despite its preference for cinnamaldehyde, will reduce aliphatic aldehydes (acetaldehyde, butyraldehyde and propionaldehyde) albeit poorly. It is worth noting that HpSCADH was capable of reducing pyridine aldehydes which may indicate that while it will oxidize aliphatic aldehydes entering the cell it may also have a specific role in an as yet unidentified metabolic processes.

While this study indicates that the organism is capable of producing a wide range of aldehydes by oxidation of the corresponding alcohol it is important to note that the reaction catalyzed by these enzymes is freely reversible and that the equilibrium for the reaction lies towards alcohol formation. Thus, it seems unlikely that such aldehydes could accumulate in the bacterium.

4.3. Inhibition studies

HpSCADH was found to be inhibited by various molecules (Table 3). By far the most significant inhibition was observed with pyrazole. This proved to be a potent competitive inhibitor of HpSCADH. While pyrazole is best known as an inhibitor of the medium-chain Zn containing alcohol dehydrogenases (see Shafqat et al., 1999) it has been shown to inhibit the Drosophila short chain alcohol dehydrogenase in a similar manner to the inhibition seen here for HpSCADH (Winberg et al., 1999).

The reduced growth profiles of the wild type *H. pylori*, in the presence of micromolar concentrations of pyrazole, suggest that this growth inhibitory effect is mediated through the inhibition of the HpSCADH enzyme.

Interestingly, Copeland et al. (2000) reported the synthesis of a class of pyrazole-based compounds that are amongst the most selective and potent inhibitors of *H. pylori* growth described to date. These compounds were designed to target dihydroorotate dehydrogenase (DHODase), an enzyme of the pyrimidine biosynthesis pathway of *H. pylori*. This raises the possibility that pyrazole and its derivatives might be combined to target *both* HpSCADH and DHODase. Since they target different pathways their growth inhibition might be expected to be additive. Further experiments are required to investigate this possibility. Nonetheless pyrazole inhibition may be useful as an adjunct to other measures to eradicate *H. pylori* since pyrazole has a long history of use in humans.

4.4. Isogenic mutant

Initially, it was believed that the ability of *H. pylori* to grow in an acidic environment was solely due to the activity of the urease enzyme. The ammonia generated from urease-mediated hydrolysis of urea was thought to neutralize stomach acid, at least in the microenvironment immediately surrounding the bacterium. However, a report in 2005 described the isolation of a urease-negative strain able to colonize the gastric mucosa suggesting other mechanisms are involved (Mine et al., 2005). Previously, we reported that HpAKR is required for optimum growth under acidic conditions (Cornally et al., 2008).

Here, we have characterized the HpSCADH negative isogenic mutant of *H. pylori* in terms of growth at both neutral and acidic pH. The data implies a role for HpSCADH in growth over extended periods under these conditions. Impairment of growth of the HpSCADH isogenic mutant may arise for a number of reasons. For example, loss of this enzyme may mean that the cell is unable to utilize specific nutrients or to metabolize toxic aldehydes. The process of acid adaptation is undoubtedly a complex process and must be effected quite quickly in order to colonize the gastric mucosa. It is possible that this process produces a number of alcohols or aldehydes that need to be metabolized rapidly. However, it is worth noting that growth was not completely halted indicating that compensatory mechanisms that overcome the loss of this

enzyme are present presumably the other alcohol dehydrogenases of the cell.

When we examined the mutant under acid stress the difference in growth became more pronounced. The pH of the gastric mucosa is believed to vary between pH 4.0 and 6.5 (Bijlsma et al., 2002). Indeed, it has been suggested that *H. pylori* is exposed to the occasional acid shock as low as pH 2.0 (see Bijlsma et al., 2002; Schreiber et al., 2005). Lower pH studies were not pursued but it may be that this growth is even further compromised at pH values below 5.0.

For the HpAKR mutant previously studied in this laboratory, no difference in growth rate between the mutant and the wild type was seen at pH 7.0 (Cornally et al., 2008). At reduced pH (pH 5 and 5.5), the difference in the growth profiles of the wild type and the knockout of HpAKR (Cornally et al., 2008) were more dramatic than those observed for the HpSCADH mutant (this study). It is possible that both HpAKR and HpSCADH play a significant role in the long-term colonization of gastric mucosa. Bijlsma et al. (2000) showed that acid resistance in *H. pylori* is a complex function of a number of expressed proteins. The present data indicate that HpSCADH contributes to this process.

5. Conclusion

In conclusion, this work shows the Hp0357 gene product (HpSCADH) to be an active short chain alcohol dehydrogenase that differs in specificity from the other two oxidoreductases from *H. pylori* in having single cofactor preference (NADH) and exhibiting a preference for aliphatic aldehydes. This work also assigns HpSCADH as a classical short chain alcohol dehydrogenase. Characterization of HpSCADH provides a more complete picture of all the aldehyde/alcohol metabolizing oxidoreductases present in *H. pylori*, demonstrating that a wide range of toxic aldehydes produced as a result of metabolism of dietary alcohols, can be efficiently reduced.

The isogenic HpSCADH negative mutant showed that under the conditions used in this study, HpSCADH is important for the growth of *H. pylori*. However, the knockout did not lead to complete inhibition of growth which implies that the function of this enzyme can be compensated by other enzymes, albeit less efficiently.

It would be of interest to determine whether the knockout of the HpAKR in conjunction with HpSCADH might be sufficient to halt growth of the organism *i.e.,* whether the inhibitory effect seen with knockout of these enzymes is additive and sufficient to justify targeting these enzymes as potential therapeutics.

In recent studies, targeting the dihydroorotate dehydrogenase enzyme of the pyrimidine biosynthesis pathway (Copeland et al., 2000) has been pursued extensively as a means to inhibit *H. pylori* growth. Significantly, pyrazole is a known therapeutic with a history of use in humans while a number of AKR inhibitors have been developed for use in humans. It is possible that a combination of such existing alcohol dehydrogenase inhibiting compounds might be sufficient to prevent *H. pylori* colonisation of gastric mucosa. The advantage of such an approach is that the therapeutics for alcohol dehydrogenase inhibition is known compounds. *In vivo* tests are required to explore this notion further.

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