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Deletion of *Helicobacter pylori* vacuolating cytotoxin gene by introduction of directed mutagenesis

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Abstract

AIM: To construct a *vacA*-knockout *Helicobacter pylori* mutant strain, whose only difference from the wild strain is its disrupted *vacA* gene.

METHODS AND RESULTS: A clone containing kanamycin resistance gene used for homologous recombination was constructed in a directional cloning procedure into pBluescript II SK, and then transformed into *vacA*$^+$ *H pylori* by electroporation. Colonies growing on the selective media containing kanamycin were harvested for chromosomal DNA extraction, and the allelic exchange was determined by polymerase chain reactions and sequencing. Loss of vacuolating activity of the *vacA*-knockout strain was confirmed by examining the gastric cells co-cultured with cell-free supernatants from *H pylori* wild strain or the mutant.

CONCLUSION: We constructed a *vacA*-knockout strain of *H pylori* through direct mutagenesis, which creates an important precondition for the future research on virulence comparison with gene expression analysis.

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INTRODUCTION

Helicobacter pylori (*H pylori*) is a Gram-negative bacterium that colonizes the gastric mucosa of humans[1], and plays an important role in pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinomas, and gastric mucosa-associated lymphoid tissue lymphomas[2-6]. Leunk *et al* first reported in 1988 that cell-free supernatants from *H pylori* broth cultures induced striking vacuolar degeneration when added to cultured eukaryotic cells[7]. Subsequently in 1992, this effect was disclosed to be caused by a secreted toxin VacA[8].

The gene encoding the vacuolating cytotoxin has been cloned from an *H pylori* isolate, and termed *vacA*[9]. Analysis of the nucleotide sequence of the *vacA* open reading frame (ORF) suggested that *vacA* encoded a 139-kDa protoxin that has three functional domains: a 33-amino-acid N-terminal signal sequence, a mature cytotoxin domain (approximately 87kDa), and a cleaved C-terminal domain (approximately 50kDa)[10,11]. VacA could induce vacuole formation from the cell cytosol, as determined by transfection of epithelial cells with a plasmid encoding the complete 95-kDa domain of VacA[12]. These vacuoles are acidic, and their membrane contains the vacuolar ATPase proton pump and the small GTP-binding protein rab7. Therefore, they have been suggested to arise from late compartments of the endocytic pathway[13].

Over the past decade, there has been considerable effort directing toward understanding the molecular mechanisms underlying VacA action. But till now, little is known about the mechanisms of vacuole formation and other effects of VacA. In this study, using the technique of direct mutagenesis to disrupt *vacA* gene, we constructed a *vacA*-knockout *H pylori* mutant strain for the further research on virulence comparison between the *H pylori* wild strain and the mutant.

MATERIALS AND METHODS

Bacterial strain and growth conditions

H pylori NCTC 11638 as a gift from Dr. Tong Shi (Shanghai Institute of Digestive Diseases) was cultured routinely on brain heart infusion (BHI) agar plates with 5 % sheep blood in an environment containing 6 % CO_2 at 37 ℃. For the preparation of cell-free supernatants from *H pylori* broth cultures, *H pylori* was cultured in BHI broth+10 % fetal bovine serum (FBS) in an environment containing 6 % CO_2 at 37 ℃ with agitation (200 rpm) for 48 h. The cultures were centrifuged (15 000 g, 30 min, 4 ℃) and filtrated with a 0.2 μm syringe filter.

Disruption of vacA gene

The strategy for disruption of *vacA* gene by direct mutagenesis is shown in Figure 1, and genetic techniques involved were described as follows.

DNA isolation To isolate chromosomal DNA, *H pylori* cells were lysed in lysis buffer (10 mM Tris· HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5 % (w/v) SDS, 20 μg/ml DNase free pancreatic RNase), and then, protease K (Sangon, Shanghai, China) was added in to a final concentration of 100 μg/ml. The lysate was incubated in a water bath at 50 ℃ for 3 h. Then, the solution was cooled to room temperature, and mixed with an equal volume of phenol equilibrated with 0.1 M Tris· HCl (pH 8.0). The two phases were separated by centrifugation at 5 000 g for 15 min at room temperature, and the aqueous phase was extracted with phenol twice again. Afterwards, 0.2 volume of 10 M ammonium acetate and 2 volume of ethanol were added to the aqueous phase. The precipitate was collected by centrifugation at 5 000 g for 2 min, washed twice with 70 % ethanol, and dissolved in an appropriate volume of TE buffer (pH8.0)[14].

Polymerase chain reactions (PCR) PCR was carried out in 100 μl volume containing 100 ng of genomic DNA, 1 U of Ex Taq (Takara), 50 pmol of each primer, and 10 nmol of each deoxynucleoside triphosphate in a standard buffer. Oligonucleotide primers (5'-CGTGGAAATCTTATTACT CTTAGC-3' and 5'-TGATGCTGACTAATGCTCCT-3')

were used to amplify a 1.7 kb product from *H pylori* NCTC 11 638. Primers for amplifying kanamycin resistance gene (*kanR*) and two fragments flanking *kanR*, *LA* and *RA*, were designed as shown in Table 1.

Gel purification and enzyme digestion PCR products were electrophoresed and excised from a 1 % agarose gel, purified using a Qiaquick gel extraction kit (Qiagen, Hilden, Germany), and digested with corresponding restriction enzymes (Promega, Madison, USA) depending on different restriction sites.

Cloning of different DNA fragments Purified PCR products for sequencing were cloned into *pGEM-T* vector (Promega). Fragments *kanR*, *LA*, and *RA* with different restriction sites on both sides were digested with corresponding endonucleases (Promega), and then cloned into *pBluescript SK* II digested with the same enzymes.

Sequencing Every clone was sequenced with the ABI DNA sequencer (Bioasia Biotechnology Company, Shanghai, China).

***H pylori* DNA transformation by electroporation** *H pylori* NCTC 11 638 cells were transformed with plasmid *pLKR* by electroporation, and kanamycin-resistant (*Km*^{r}) transformants were selected by a method similar to that described by Clayton *et al*[15]. Briefly, *H pylori* cultured on plates were scraped and suspended in 30 ml cold double-distilled water. Cells were harvested by centrifugation at 4 360 g at 4 ℃ for 5 min, and the pellet was suspended in 20 ml of cold 10 % glycerol. The cells were centrifuged once, and resuspended in 2 ml ice-cold 10 % glycerol. Plasmid DNA (1 μg in 5 μl TE buffer) was mixed with 0.2 ml cell suspension. The mixture was added to a prechilled (-20 ℃) 0.2 cm electroporation cuvet (Bio-Rad, Hercules, USA), and subjected to single-pulse electroporation of initial voltage 2.5 kV, 25 μF and 600Ω in parallel. The sample was transferred onto a cold plate and incubated for 12 h at 37 ℃. Then the cells were inoculated onto selective media with 30 μg/mL kanamycin, followed by incubation for 4 d to allow the growth of transformants.

Cell culture and detection of vacuole formation

Cells of gastric cancer cell line SGC7901 as a gift from Jie Yang (Department of Cell Biology, Shanghai Second Medical University) were grown in DMEM (GIBCO-BRL, Gaithersburg, USA) supplemented with 10 % FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5 % CO_2 atmosphere. SGC7901 cells were co-cultured with cell-free supernatants from *H pylori* NCTC11638 wild strain or mutant strain for 12 h, and then observed by contrast microscopy.

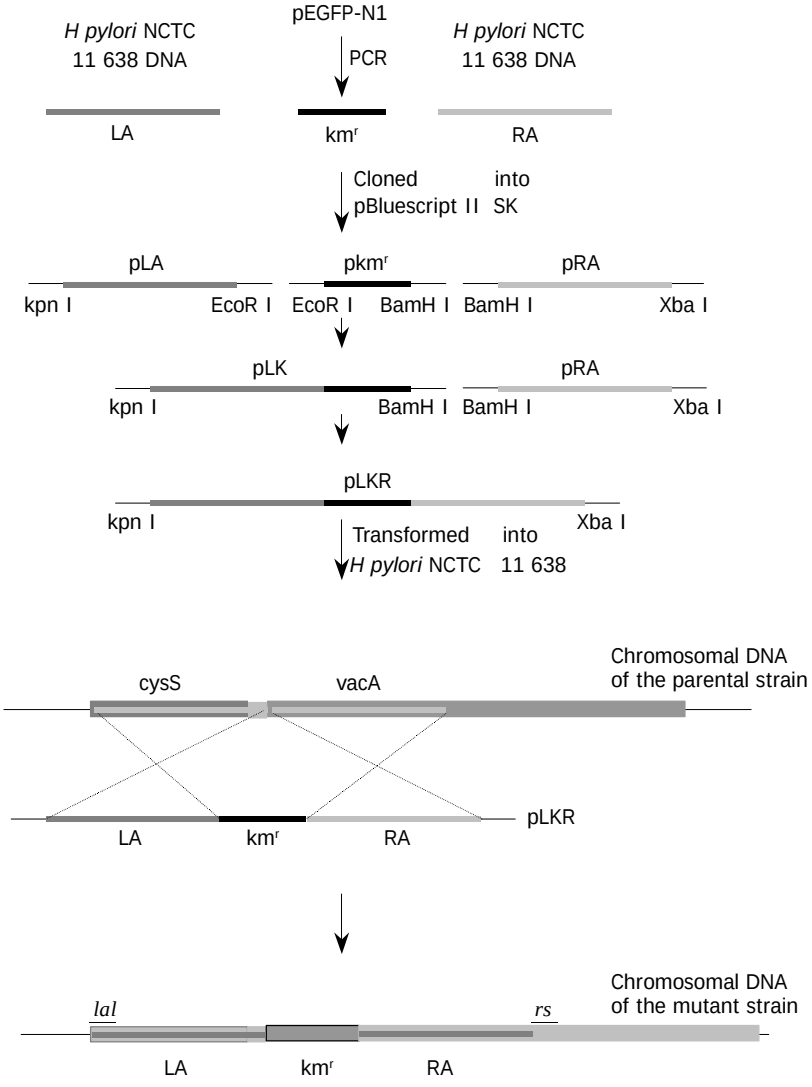

Figure 1 The strategy for disruption of *vacA* gene by directed mutagenesis. LA and RA were PCR-amplified from *H pylori* NCTC 11638 genomic DNA, and the kanamycin resistance gene, from pEGFP-N1. PCR products with different restriction sites on both sides were digested with corresponding endonucleases, and then cloned into pBluescript II SK digested with the same enzymes. Because there is an *EcoR* II site in RA, *pLA* and *pkm*^{r} were firstly joined together, resulting in *pLK*, which then was joined with *pRA*, resulting in *pLKR*. The plasmid *pLKR* was transformed into *H. pyloi* NCTC 11638, where the *Km*^{r} marked mutation was introduced into the genome by homologous recombination, resulting in the *vacA*$^-$Km^r mutant strain.

Table 1 Primers for amplifying *km*^{r}, LA and RA

Primer	Sequence (5' to 3')	Site	Coordinates
la1	GGGGTACCCTTTTGAGCCTTTAGTT	*Kpn* I	cysS bp36-54
la2	CGGAATTCTCCTTTCTTTTTGTAAAAC	*Eco*R I	HPU07145 bp382-400
km^r*1*	CGGAATTCATGATTGAACAAGATGGATTG	*Eco*R I	pEGFP-N1 bp 2629-2649
km^r*2*	CGGGATCCTCAGAAGAACTCGTCAAG	*Bam*H I	pEGFP-N1 bp3406-3423
ra1	CGGGATCCATCGCCCTCTGGTTTCTC	*Bam*H I	HPU07145 bp 437-454
ra2	GCTCTAGACACCCACTTGATTATTCACTCT	*Xba* I	HPU07145 bp 1786-1807

cysS *la1*

1 ATGTTTATTT ATGATACCAA ATTAAAACAA AAAGTCCCTT TTGAGCCTTT

51 AGTTGAAAAA AAGGCGAATA TTTATGTGTG CGGGCCTACG GTGTATGATG
101 ACGCTCATTT AGGGCATGCC AGGAGCGCGA TTGCTTTTGA TTTGTTAAGG
151 CGCACGCTTG AATTGAGCGG CTATGAAGTG GTGTTAGTAA GGAATTTCAC
201 GGATATTGAC GATAAAATCA TCAACAAAGC CTTAAAAGAA AACAAAAGCA
251 TTCAAGAATT AAGCAGCATT TACATTGAAT CTTACACGAG GGATTTAAAC
301 GCTTTGAACG TGAAAAAACC CAGCCTAGAG CCTAAAGCGA GCGAGTATTT
351 AGACGCTATG GTGGGCATGA TTGAAACGCT TTTAGAAAAA AATATCGCTT
401 ATCAGGTCTC TAATGGGGAT ATTTATTTAG ACACGAGCAA GGATAAAGAT
451 TACGGCTCTT TGAGCGTGCA TAATAGCAGT ATGGAATTTG GCCGTATTGG
501 TTTGGTGCAA GAAAAACGGC TTGAGCAGGA TTTTGTGCTA TGGAAAAGCT
551 ATAAGGGGGA TAATGATGTG GGTTTTGATA GCCCTTTAGG CAAAGGGCGC
601 CCTGGCTGGC ATATAGAATG CTCTAGCATG GTTTTTGAAA CTTTAGCACT
651 CGCTAACACC CCTTATCAAA TTGACATCCA TGCAGGCGGA GCGGATCTGT
701 TATTCCCCCA CCATGAAAAT GAAGCGTGCC AAACCCGTTG CACCTTTGGC
751 GTGGAGCTTG CTAAATACTG GATGCATAAT GGCTTTGTGA ATATCAACAA
801 CGAAAAAATG TCTAAAAGTT TAGGGAATAG CTTTTTTATT AAAGACGCCC
851 TGAAAAACTA TGATGGCGAG ATTTTGCGCA ATTATTTACT AGGGGTGCAT
901 TATCGCTCTG TTTTGAATTT CAATGAAGAA GACTTGTTAG TGAGTAAAAA
951 ACGCTTGGAT AAAATCTATC GTTTGAAACA GCGCGTTTTA GGGACTTTGG
1001 GAGGAATAAA TCCAAACTTT AAAAAAGAAA TTTTAGAGTG CATGCAAGAT
1051 GATTTAAACG TTTCTAAAGC GTTGAGCGTT TTAGAAAGCA TGCTTTCTTC
1101 TACGAATGAA AAACTGGATC AAAACCCCAA AAACAAGGCT TTGAAGGGCG
1151 AAATTTTAGC GAATTTGAAA TTCATAGAAG AACTGCTTGG TATTGGGTTT
1201 AAAGACCCTA GCGCGTATTT CCAGTTAGGC GTGAGCGAGA GCGAAAAACA
1251 AGAAATTGAA AACAAGATAG AAGAAAGAAA ACGCGCCAAA GAACAAAAAA
1301 TTTTTTTAAA AGCCGATAGC ATCAGAGAAG AACTTTTGAA ACAAAAAATC
1351 GCTTTGATGG ACACCCCACA AGGCACGATT TGGGAGAAGT TTTTTTAAAC
1401 GCCTCCAATT TTACCTTTTT ACACATTCTA GTAACAACCT TCAGCATTTT
1451 TGCTTTTTAA TCTTGTTAAG TTTTATGTTC ATTTACTTTA ATTTGATAAA

1501 AATTGAACAT TGGTTGTAGA TACTATATAT TTATAGCCTT AATCGTAAAT
-35 signal -10 signal

1551 GCAACAGAAA TTTTCTAGTC TAAAGTCGCA CCCTTTGTGC AAAAATCGTT

la2 km^r

1601 TTACAAAAAG AAAGGAGAAT TCATGATTGA ACAAGATGGA TTGCACGCAG
rbs

1651 GTTCTCCGGC CGCTTGGGTG GAGAGGCTAT TCGGCTATGA CTGGGCACAA
1701 CAGACAATCG GCTGCTCTGA TGCCGCCGTG TTCCGGCTGT CAGCGCAGGG

1751 GCGCCCGGTT CTTTTTGTCA AGACCGACCT GTCCGGTGCC CTGAATGAAC
1801 TGCAAGACGA GGCAGCGCGG CTATCGTGGC TGGCCACGAC GGGCGTTCCT
1851 TGCGCAGCTG TGCTCGACGT TGTCACTGAG GCGGGAAGGG ACTGGCTGCT
1901 ATTGGGCGAA GTGCCGGGGC AGGATCTCCT GTCATCTCAC CTTGCTCCTG
1951 CCGAGAAAGT ATCCATCATG GCTGATGCAA TGCGGCGGCT GCATACGCTT
2001 GATCCGGCTA CCTGCCCATT CGACCACCAA GCGAAACATC GCATCGAGCG
2051 AGCACGTACT CGGATGGAAG CCGGTCTTGT CGATCAGGAT GATCTGGACG
2101 AAGAGCATCA GGGGCTCGCG CCAGCCGAAC TGTTCGCCAG GCTCAAGGCG
2151 AGCATGCCCG ACGGCGAGGA TCTCGTCGTG ACCCATGGCG ATGCCTGCTT
2201 GCCGAATATC ATGGTGGAAA ATGGCCGCTT TTCTGGATTC ATCGACTGTG
2251 GCCGGCTGGG TGTGGCGGAC CGCTATCAGG ACATAGCGTT GGCTACCCGT
2301 GATATTGCTG AAGAGCTTGG CGGCGAATGG GCTGACCGCT TCCTCGTGCT
2351 TTACGGTATC GCCGCTCCCG ATTCGCAGCG CATCGCCTTC TATCGCCTTC
ra1
2401 TTGACGAGTT CTTCTGAGGA TCCATCGCCC TCTGGTTTCT CTCGCTTTAG

2451 TAGGAGCATT AGTCAGCATC ACACCGCAAC AAAGTCATGC CGCCTTTTTC
2501 ACAACCGTGA TCATTCCAGC CATTGTTGGG GGTATCGCTA CAGGCACCGC
2551 TGTAGGAACG GTCTCAGGGC TTCTTAGCTG GGGGCTCAAA CAAGCCGAAG
2601 AAGCCAATAA AACCCCAGAT AAACCCGATA AAGTTTGGCG CATTCAAGCA
2651 GGAAAAGGCT TTAATGAATT CCCTAACAAG GAATACGACT TATACAGATC
2701 CCTTTTATCC AGTAAGATTG ATGGAGGTTG GGATTGGGGG AATGCCGCTA
2751 GGCATTATTG GGTCAAAGGC GGGCAACAGA ATAAGCTTGA AGTGGATATG
2801 AAAGACGCTG TAGGGACTTA TACCTTATCA GGGCTTAGAA ACTTTACTGG
2851 TGGGGATTTA GATGTCAATA TGCAAAAAGC CACTTTACGC TTGGGCCAAT
2901 TCAATGGCAA TTCTTTTACA AGCTATAAGG ATAGTGCTGA TCGCACCACG
2951 AGAGTGGATT TCAACGCTAA AAATATCTCA ATTGATAATT TTGTAGAAAT
3001 CAACAATCGT GTGGGTTCTG GAGCCGGGAG GAAAGCCAGC TCTACGGTTT
3051 TGACTTTGCA AGCTTCAGAA GGGATCACTA GCGATAAAAA CGCTGAAATT
3101 TCTCTTTATG ATGGTGCCAC GCTCAATTTG GCTTCAAGCA GCGTTAAATT
3151 AATGGGTAAT GTGTGGATGG GCCGTTTGCA ATACGTGGGA GCGTATTTGG
3201 CCCCTTCATA CAGCACGATA AACACTTCAA AAGTAACAGG GGAAGTGAAT
3251 TTTAACCACC TCACTGTTGG CGATAAAAAC GCCGCTCAAG CGGGCATTAT
3301 CGCTAATAAA AAGACTAATA TTGGCACACT GGATTTGTGG CAAAGCGCCG
3351 GGTTAAACAT TATCGCTCCT CCAGAAGGTG GCTATAAGGA TAAACCCAAT
3401 AATACCCCTT CTCAAAGTGG TGCTAAAAAC GACAAAAATG AAAGCGCTAA
3451 AAACGACAAA CAAGAGAGCA GTCAAAATAA TAGTAACACT CAGGTCATTA
3501 ACCCACCCAA TAGTGCGCAA AAAACAGAAG TTCAACCCAC GCAAGTCATT
3551 GATGGGCCTT TTGCGGGCGG CAAAGACACG GTTGTCAATA TCAACCGCAT
3601 CAACACTAAC GCTGATGGCA CGATTAGAGT GGGAGGGTTT AAAGCTTCTC
3651 TTACCACCAA TGCGGCTCAT TTGCATATCG GCAAAGGCGG TGTCAATCTG
3701 TCCAATCAAG CGAGCGGGCG CTCTCTTATA GTGGAAAATC TAACTGGGAA
ra2
3751 TATCACCGTT GATGGGCCTT TAAGAGTGAA TAATCAAGTG GGTGGCTATG

rs
3801 CTTTGGCAGG ATCAAGCGC

Figure 2 Nucleotide sequence of cysS gene and the downstream sequence amplified from the *vacA*$^{-}$*Km*^{r} mutant *H pylori*. The 1 398 bp *cysS* ORF and the 795 bp *km*^{r} ORF are shown. Primers *la1*, *la2*, *ra1*, *ra2*, and *rs* for amplifying LA, RA, and ASm are indicated. -35 signal, -10 signal, and rbs of *vacA* gene serving km^{r} gene in the mutant strain are also shown.

RESULTS

Upstream sequence close to vacA *gene*

Genome of NCTC 11638 was not completely sequenced, and the upstream portion close to *vacA* gene going to be used in the mutagenesis technique was not published in GeneBank. Therefore, the upstream sequences close to *vacA* gene of 26695 and J99, whose genomes were completely sequenced and published, were aligned and searched for conservative sequences. Then a 1.7-kb product was PCR-amplified from *H pylori* NCTC11638 DNA and sequenced. The sequencing result showed the complete *cysS* (cysteinyl-tRNA synthetase) gene of NCTC 11638 (Figure 2).

Cloning of pLKR for transforming H pylori

As shown in Figure 1, LA which contains the *H pylori vacA* promoter and RA were amplified from genomic DNA of NCTC11638, while *kanR* gene which has no promoter was amplified from the plasmid *pEGFP-N1* (Clontech, Palo Alto, USA). PCR-products LA, *kanR* and RA, with restriction sites incorporated at the termini, were joined together in a directional cloning procedure into *pBluescript* II *SK*, resulting in *pLKR*.

Construction of vacA-knockout H pylori mutant strain

pLKR which is unable to replicate in *H pylori*, was introduced into *H pylori* NCTC11638 by electroporation. After 4 d of growth, five Km^r single colonies were isolated. To determine whether *vacA* had been disrupted in the transformed strains through allelic exchange, DNA isolated from *H pylori* NCTC11638 wild-type strain and the Km^r mutant strain were PCR-amplified with the primers *la1* and *rs* (5' -GCGCTTGATC CTGCCAAAGCATAGC-3') annealing to *H pylori* NCTC 11638 *vacA* at bp 1808 to 1832 flanking *ra2* (Figures 1 and 2). A 3.8-kb product consistent with the expected size was PCR-amplified from Km^r mutant strain, as compared with a 3.0-kb product amplified from wild strain (Figure 3), suggesting a substitution of Km^r gene for a short fragment of *vacA* gene by homologous recombination between plasmid and chromosomal sequences. The sequencing result of the 3.8-kb product confirmed the occurrence of allelic exchange (Figure 2).

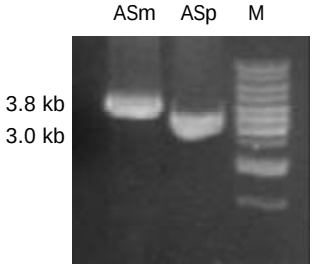

Figure 3 PCR amplification for the determination of homologous recombination in Km^r mutant strain. Genomic DNA of NCTC 11638 wild strain and Km^r mutant strain were respectively PCR-amplified for the fragments ASm and ASp using the primers *ra1* and *rs*. A single 3.8 kb product ASm was amplified from Km^r mutant strain as compared with the 3.0 kb product ASp amplified from the wild strain.

Characterization of vacA-knockout H pylori mutant

To determine the loss of vacuolating activity of the mutant strain, gastric cells SGC7901 were co-cultured with cell-free supernatants from *H pylori* NCTC11638 wild strain or mutant strain for 12 h, and then observed by contrast microscopy. Intracellular vacuoles developed in cells co-cultured with supernatant from the wild strain, while no vacuoles developed in cells co-cultured with supernatant from $vacA^-Km^r$ mutant strain (Figure 4).

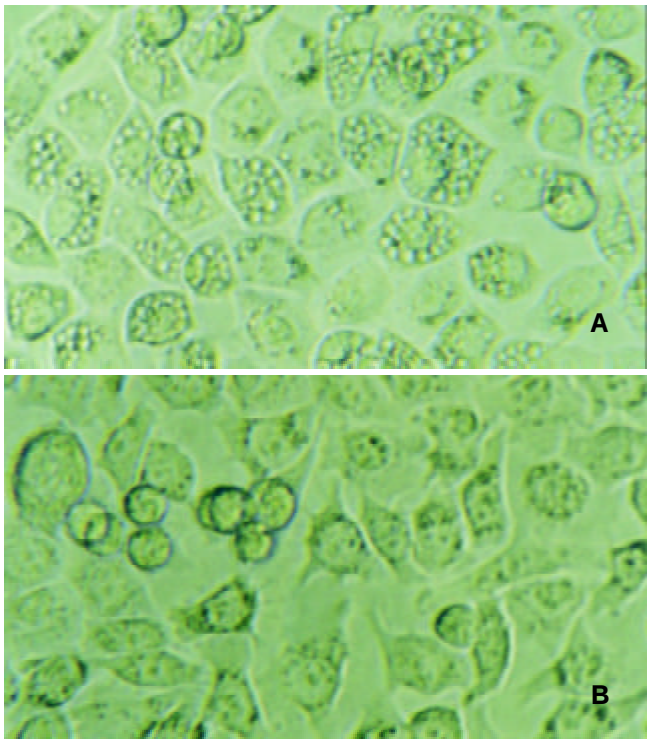

Figure 4 Gastric cancer cells SGC-7901 were co-cultured with the supernatant either from *Helicobacter pylori* NCTC 11638 or its *vacA*-knockout mutant strain. 12 h after the incubation, A: cells co-cultured with NCTC 11638 developed vacuoles in the cytosol; B: cells co-cultured with $vacA^-$ mutant strain developed no vacuoles at all.

DISCUSSION

VacA, produced by pathogenic strains of *H pylori*, was a major virulence factor in pathogenesis of gastroduodenal ulcers[8,16]. VacA induced the formation of membrane-delimited vacuoles in intoxicated cells[7], and showed many other effects on cellular functions and viability, such as causing mitochondrial depolarization[17], inducing apoptosis in gastric cells[18], affecting or interacting with various components of cytoskeleton to cause actin rearrangements[19], and even disorganizing microtubular network[20]. To study VacA machanism of action, we have tried to get purified VacA used as single virulence determinant, to study its effect on the expression profile of eukaryocyte. However, like Manetti *et al*[21], we did not successfully get the expressed VacA as a functional recombinant protein in *E. coli*, probably due to its incorrect fold. We have also considered letting VacA directly expressed in the cytosol to induce vacuole formation. In our experiments, vacuoles were induced in only 10 % of cells transfected with plasmids expressing VacA, because the efficiency of the transfection method was relatively low. In addition, when VacA acts outside the cells, the pathway by which it interacts with the cells is quite different from that when the protein is produced in the cytosol. Under natural conditions, association of VacA with the eukaryotic cell surface was the first step in the intoxication of cells[22]. The initial interaction of VacA with target cells was through high-affinity cell surface receptors, and this interaction was necessary for its biologic activity[23,24]. A 250 kDa receptor protein tyrosine phosphatase (RPTP) β served as a receptor for VacA on AZ521 cells, and another protein, p140, was also commonly detected in VacA-susceptible cells[25,26]. Increased binding of acid- or alkali-activated VacA to RPTPβ may alter its activity and possibly accelerates or inhibits dephosphorylation of tyrosine on cytosolic proteins. Moreover, VacA acting outside the cells

is a kind of exogenous antigen, having different pathways of processing and presentation from that of VacA expressed in the cytosol as an endogenous antigen. All of these processes may affect gene expression of the host cells.

Direct mutagenesis was probably the most useful technique for assessing the contribution to virulence of specific bacterial gene products[27]. In our study, *vacA* gene encoding vacuolating cytotoxin that has been identified by conventional biochemical means was disrupted by gene replacement. This technique requires a means for introducing DNA into the pathogen, as well as suitable selective markers and an inherent capacity for homologous recombination. In previous studies, the Km^r determinant often came from *Campylobacter coli*[28]. Here we introduced a simple method to get the Km^r gene from commercialized plasmids such as *pEGFP-N1*. Coding sequence of the gene without a promotor was PCR-amplified from *pEGFP-N1* and ligated downstream with the promotor of *H pylori vacA* gene. Upon insertion into chromosomal DNA of *H pylori* through homologous recombination, this gene could be efficiently transcribed because the *vacA* promoter was recognized by *H pylori* transcriptional machinery, introducing kanamycin resistance characteristics into *H pylori*. Due to the stop coden of Km^r, *vacA* would not be translated at all although most of the sequences still existed. The results of PCR and sequencing confirmed the occurrence of allelic exchange. Therefore, using the direct mutagenesis technique, we obtained the isogenic mutant strain of *H pylori*, which differed from the wild strain only in that the *vacA* gene was knocked out. Through co-culture of cell-free supernatants from the wild or mutant *H pylori* strain with gastric cells, loss of vacuolating activity of the *vacA*-knockout strain was confirmed. These results clearly show that VacA is an indispensable toxin secreted by *H pylori* for the induction of vacuole formation.

Such kind of technique has been used to yield $vacA^-$ mutant *H pylori*[9,28,29]. But no further experiment has been done to compare the virulence between the mutant and the parental strain. On the other hand, microarray analysis has been used in several studies to screen gene expression profiles in gastric epithelial cells induced by *H pylori*[30-32]. Our group has also analyzed different expression profiles of gastric cancer cells co-cultured with supernatants of $VacA^+$ or $VacA^-$ *H pylori* isolates. However, VacA has not been used as a single virulence determinant to stimulate host cells, thus one can not determine which virulent factors result in the alteration of the expression. In this study, we successfully constructed the $vacA^-$ mutant strain, using the direct mutagenesis technique, which creates an important precondition for the further research on virulence comparison with gene expression analysis.

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