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ORIGINAL ARTICLE

Characterization of an *Helicobacter pylori* environmental strain

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Abstract

Aims: To investigate the main genotypic virulence markers and the phenotypic features of an environmental *Helicobacter pylori* strain, named MDC1.
Methods and Results: The *H. pylori* MDC1 genotypic status was evaluated by PCR amplification. The mosaicism in *vac*A alleles was expressed by the s1m1 allelic combination, as found in strains which are strong vacuolating cytotoxin producers; the number of *cag*A variable EPIYA motifs displayed P1P2P3P3 pattern and the *ice*A1 was recorded between the *ice*A allelic types and the *bab*A2 gene found in strains causing more severe disease. The biofilm formation was evaluated on a polystyrene surface in static conditions by scanning electron microscopy and confocal scanning laser microscopy. *Helicobacter pylori* MDC1 displayed a dense mature biofilm with cells in a coccoid morphology persistent in time in which the expression of the *lux*S gene, related to the quorum-sensing signalling, was always detected.
Conclusions: *Helicobacter pylori* MDC1 strain had the main virulence markers closely related to gastric pathogenesis and displayed a well-structured biofilm which allowed this bacterium to be more protected in the environment.
Significance and Impact of the Study: The persistence of the environmental virulent *H. pylori* strain in a clustered state suggests a long-term survival of this bacterial community outside of the host, enabling the bacterial transmission with important clinical repercussions.

Introduction

Helicobacter pylori is estimated to infect the gastric mucosa of half of the world population, representing the causative agent of gastritis and peptic ulcer diseases, and it has also been described as a risk factor for gastric carcinoma (Suerbaum and Michetti 2002; van Amsterdam *et al.* 2006). The natural habitat for the micro-organism is the human stomach, but it may also survive in other environments to become a life-long infection threat (Sasaki *et al.* 1999). *Helicobacter pylori* has been detected in dental plaque, in human and animal faeces (Grubel *et al.* 1997; Parsonnet *et al.* 1999; Kabir 2003), and a large number of studies report its presence in aquatic environments (Adams *et al.* 2003; Cellini *et al.* 2004, 2005a; Braganca *et al.* 2007). Water supplies contaminated by sewage containing fluids or faeces from infected people have therefore been considered as a potential source of *H. pylori* transmission (Mazari-Hiriart *et al.* 2001; Park *et al.* 2001; Lu *et al.* 2002; Cellini *et al.* 2004; Ahmed *et al.* 2007).

Helicobacter pylori is characterized by a dynamic behaviour aimed to express real strategies for balancing its spread both outside and inside the host. In fact, it has been demonstrated that *H. pylori* has the ability of forming biofilm as a strategy to overcome environmental stress and to protect itself (Stark *et al.* 1999; Cole *et al.* 2004; Cellini *et al.* 2005b; Azevedo *et al.* 2006; Carron *et al.* 2006; Coticchia *et al.* 2006).

In a previous study, *H. pylori* was isolated by culture from marine zooplankton supporting speculation about the potential role of zooplankton in *H. pylori* survival and

transmission (Cellini *et al.* 2005a). In that study, the isolated micro-organism, named *H. pylori* MDC1, was characterized by standard microbiological tests, by PCR amplification of the species-specific target gene *vac*A and *cag*A fragments, and by comparative sequence analysis of the amplified *vac*A gene products. The aim of the present study was to investigate the genotypic and phenotypic *H. pylori* MDC1 features.

The micro-organism was especially studied for its genotypic status to assess its potential virulence. To do this, the following had to be detected: (i) the mosaic combination of s and m allelic variants of the *vac*A gene which are related to the production of vacuolating cytotoxin (Atherton *et al.* 1995); (ii) the presence of the number of *cag*A variable region EPIYA motifs which are more closely related to gastric carcinogenesis (Argent *et al.* 2005); (iii) the presence of the *ice*A1/A2 genes related to the IL-8 production (van Doorn *et al.* 1998); (iv) the gene encoding blood group antigen-binding adhesin (BabA), *bab*A2, which is a useful marker to identify patients who are at higher risk for peptic ulceration and gastric adenocarcinoma (Mizushima *et al.* 2001).

The environmental strain was, finally, phenotypically analysed through the evaluation of its sessile biofilm lifestyle, which is particularly documented among micro-organisms living in aqueous environments (Moss *et al.* 2006). The *H. pylori* MDC1 biofilm formation was investigated, over time, through microscopic observations to assess the morphology and the viability of the microbial community, and through molecular analysis evaluating the expression of the *lux*S gene, which is related to the quorum-sensing signalling (Schauder *et al.* 2001) and represents a significant indicator of biofilm production (Cole *et al.* 2004).

Materials and methods

Helicobacter pylori culture

Helicobacter pylori MDC1 was the environmental strain used for the experiments. The strain was isolated in fractionated seawater samples collected in the Adriatic sea at a depth of 5 m at about 500 m from the coast of Pescara, Italy, bound to large zooplanktonic organisms (Cellini *et al.* 2005a). The micro-organisms were identified by standard biochemical tests (urease, catalase, oxidase) and by molecular methods by using target *vac*A and *cag*A genes. In particular, the species-specific amplified 537 bp *vac*A fragment which was 85 bp smaller in size than the standard control strain *H. pylori* ATCC 43629, was sequenced and deposited in the NCBI database (Genbank accession number: AY 848858). According to the method of Drumm and Sherman (1989), the strain was stored at −80°C until it was thawed at room temperature, plated rapidly on chocolate agar (CA) plus 1% (v/v) IsoVitaleX (Becton Dickinson, Buccinasco, Italy) and incubated at 37°C for 5–7 days in a microaerophylic atmosphere (Campy Pak Jar; Oxoid Ltd, Italy).

Virulence factor genotyping

The chromosomal DNA was extracted from *H. pylori* MDC1 colonies using Qiamp Tissue DNA isolation mini-kit (Qiagen SpA, Milano, Italy). PCR reactions to evaluate the genotyping *H. pylori* MDC1 status were carried out in a 2700 Thermocycler (PE-Applied Biosystems, Foster City, CA, USA) with oligonucleotide primers listed in Table 1 in a total volume of 25 μl containing 2·5 μl of 10× PCR buffer, 1·5 mmol l^{-1} $MgCl_2$, 200 mmol l^{-1} (each) deoxynucleotide triphosphates (dNTP), 2 U of Amplitaq DNA polymerase, 20 mmol l^{-1} of each primer and 50 ng of *H. pylori* MDC1 DNA except for *ice*A1/A2 genes for which 10 ng of DNA was used.

Regarding the *cag*A variable-region EPIYA motifs, the determination of the number of the nucleotide sequences encoding EPIYA motifs were carried out using the forward primer cagA 28F and the reverse primers cagA-P1C, cagA-P2CG, cagA-P2TA and cagA-P3E.

Amplification consisted of 5 min of denaturation followed by 30–35 cycles as follows: 40 s at 94°C, 1 min of annealing at 55–62°C and 1 min and 30 s of extension at 72°C (for the analysis of *vac*A s/m regions, *ice*A1, *ice*A2 and *bab*A2 genes); 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for the amplification of *cag*A 3′ variable region and EPIYA phosphorylation motifs. After the last cycle, the extension was continued for 5 min. Each oligonucleotide sequence used in this work was synthesized by Primm (Milano, Italy).

The PCR products were examined by electrophoresis in 2% (w/v) agarose gel at 100 V for 30 min. Gels were stained with ethidium bromide and photographed. Experiments were performed in triplicate.

Growth of biofilm *in vitro*

For biofilm formation, which was performed as previously indicated for clinical *H. pylori* strains (Cellini *et al.* 2005b), bacteria were harvested in Brucella broth (BB; Biolife, Italy) supplemented with 2% (w/v) foetal calf serum (Biolife, Italy) and 0·3% (w/v) glucose (Sigma Aldrich, Milan, Italy). Broth cultures, gently shaken, were incubated overnight at 37°C in a micro-aerophylic atmosphere (85% N, 5% O_2, 10% CO_2). After incubation, each broth culture was adjusted to an optical density at 600 nm (OD_{600}) of approximately 0·1, which corresponded to a concentration of 5×10^3 to 8×10^3 CFU ml^{-1} of cells at

Table 1 Oligonucleotides used for PCR-based typing

Primer designation	Gene	Sequence (5′–3′)	Expected size of PCR product bp (motif amplified)	References
VA1-F	*vac*A	ATGGAAATACAACAAACACAC	259 (s1)	Chisholm *et al.* 2002
VA1-R		CTGCTTGAATGCGCCAAAC	286 (s2)	
VAG-F	*vac*A	CAATCTGTCCAATCAAGCGAG	567 (m1)	Chisholm *et al.* 2002
VAG-R		GCGTCAAAATAATTCCAAGG	642 (m2)	
cag2[a]	*cag*A	GGAACCCTAGTCGGTAATG	550–800	Rudi *et al.* 1998
cag4[a]		ATCTTTGAGCTTGTCTATCG		
cagA28F	*cag*A	TCTCAAAGGAGCAATTGGC	264–291 (P1)	Argent *et al.* 2005
cagA-P1C		GTCCTGCTTTCTTTTTATTAACTTKAGC		
cagA-P2CG	*cag*A	TTTAGCAACTTGAGCGTAAATGGG	309–336 (P2)	Argent *et al.* 2005
cagA-P2TA	*cag*A	TTTAGCAACTTGAGTATAAATGGG	309–336 (P2)	
cagA-P3E	*cag*A	ATCAATTGTAGCGTAAATGGG	465–498/672 (P3)	
iceA1-F	*ice*A	TATTTCTGGAACTTGCGCAACCTGAT	800 (A1)	Mukhopadhyay *et al.* 2000
M.Hpy1-R		GGCCTACAACCGCATGGATAT		
CysS-F	*ice*A	CGGCTGTAGGCACTAAAGCTA	750 (A2)	Mukhopadhyay *et al.* 2000
iceA2-R		TCAATCCTATGTGAAACAATGATCGTT		
babA2sense	*bab*A	AATCCAAAAAGGAGAAAAAGTATGAAA	800–900 (A2)	Gerhard *et al.* 1999
babA2antisense		TGTTAGTGATTTCGGTGTAGGACA		

the beginning of the exponential phase and inoculated into polystyrene Petri dishes (Steroglass, Perugia, Italy) 3·5 cm in diameter for scanning electron microscopy (SEM) observations, into one-well polystyrene chamber slides (Nunc, EuroClone SpA, Life-Sciences-Division, Milano, Italy) for confocal scanning laser microscopy (CSLM) observations and into polystyrene cell-culture flasks 25 cm^2 in area with a 0·2 μm vent cap (Corning, New York, USA) for biofilm molecular analysis. Cell suspensions, 2, 2·5 and 12 ml were inoculated into Petri dishes, chamber slides and cell culture flasks, respectively. Cultures were incubated at 37°C in a micro-aerophylic atmosphere, without shaking, for various times. At the desired time-point, nonadherent cells were removed, and sessile micro-organisms were rinsed with phosphate-buffered saline (PBS, pH 7·2).

SEM and CSLM observations

For SEM analysis, sessile bacteria attached to polystyrene Petri dishes were pre-fixed in a solution of 2·5% glutaraldehyde with 0·1 mol l^{-1} cacodylate buffer at pH 7·2 for 1 h and then washed with cacodylate buffer solution. After being washed again in a PBS buffer solution, the plates were post-fixed in a 2% osmium tetroxide, and dehydrated in a series of graded ethanol solutions ranging from 50% to 100%. All the samples were gold coated with sputter (Emitech K 550; Emitech Ltd, Ashford, Kent, UK).

Scanning electron microscopy examination (LEO 435 Vp, Cambridge, UK) was performed using secondary electrons (SE1) with a current probe varying from 49 to 200 pA at 20·00–25·00 keV.

Each cell referred as a pixel in the grid has been assigned a value between 0 and 255 (0 = black; 255 = white). The method used for the quantification of cellular morphology involved the measure of compactness, which is a measure of an object's circularity using a software Sigma Scan Pro 5 image analysis (Cellini *et al.* 2005b).

For CSLM analysis, sessile bacteria attached to one-well polystyrene chamber slides were investigated during *H. pylori* MDC1 biofilm development by Live/Dead *Bac*Light bacterial viability kit (Molecular Probes Inc., Invitrogene, Italy). The two stock solutions of the stain (SYTO 9 and propidium iodide) were injected into the chamber slides and the samples were observed through a Zeiss confocal microscope using the 488 nm line from an argon ion laser and 535 nm band pass emission filter. A Zeiss 40×/1·3 oil and 10×/0·3 numerical aperture objective was used for the images. Serial sections in the *x*–*y* plane were obtained at 6·3 μm intervals along the *z*-axis; three-dimensional reconstruction imaged biofilms were obtained by the resident-local software. The images were further processed for display by using PhotoDraw software (Adobe Systems Inc., San Jose, CA). Zeiss LSM510 META (Jena, Germany) confocal system was equipped with an argon laser and helium–neon source. Images were collected by Zeiss acquisition software ver 3·2. To separate the emissions of the two fluorochromes HTF 488/543 and NTF 545 primary and secondary dichroic mirrors, respectively, were used. Detector band pass filters were set over 505–530 and 565–615 ranges for the green and red emissions which were simultaneously recorded. All CSLM experiments were performed three times with similar results.

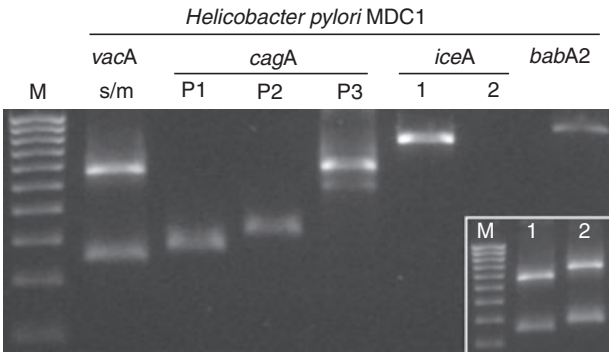


Figure 1 *vac*A, *cag*A, *ice*A and *bab*A2 genotypic status of *Helicobacter pylori* MDC1 environmental strain. DNA size standard (0·1 kbp marker) are in the lanes marked M. Insert, comparison between *vac*A s1m1 *H. pylori* MDC1 (sample 1) and *vac*A s2m2 *H. pylori* clinical strain 37/2 (sample 2) profiles.

Detection of *lux*S gene expression

For the *H. pylori* MDC1 biofilm phenotypic analysis from cell-culture flasks, bacteria attached to the polystyrene surface were removed using a cell scraper (Corning).

Bacterial RNA was extracted from sessile cells using an Rnasy Mini Kit (Qiagen, Hilden, Germany). Before performing the reverse transcriptase–polymerase chain reaction (RT–PCR), a DNase digestion was carried out. The primers used were: XP1 (5′-ATGAAAACACCAAA-AATGAATGTAG-3′) and XP2 (5′-AACCCCCACTTCA-GACCAC-3′) for *lux*S (Joyce *et al.* 2000).

Reverse transcriptase–polymerase chain reaction was performed using the One Step RT–PCR Kit (Qiagen) in a final reaction volume of 25 μl containing 50 ng of total RNA, 1× Qiagen buffer, 500 μmol l^{-1} of each dNTP, 0·6 μ mol l^{-1} of *lux*S primer and 5 U of One Step RT enzyme. RT–PCR Master Mix was performed in a 2700 thermocycler (Applied Biosystems, Foster City, CA, USA) for 30 min at 50°C and 15 min at 95°C for RT and initial PCR activation. Amplification of *lux*S cDNA consisted of 95°C for 15 min and then 30 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, with a final 10 min extension at 72°C. Samples (5 μl) of PCR products were analysed by electrophoresis in a 2% (w/v) agarose gel at 100 V for 45 min. Gels were stained with ethidium bromide and photographed. The expected size of the amplification products for *lux*S was approximately 465 bp.

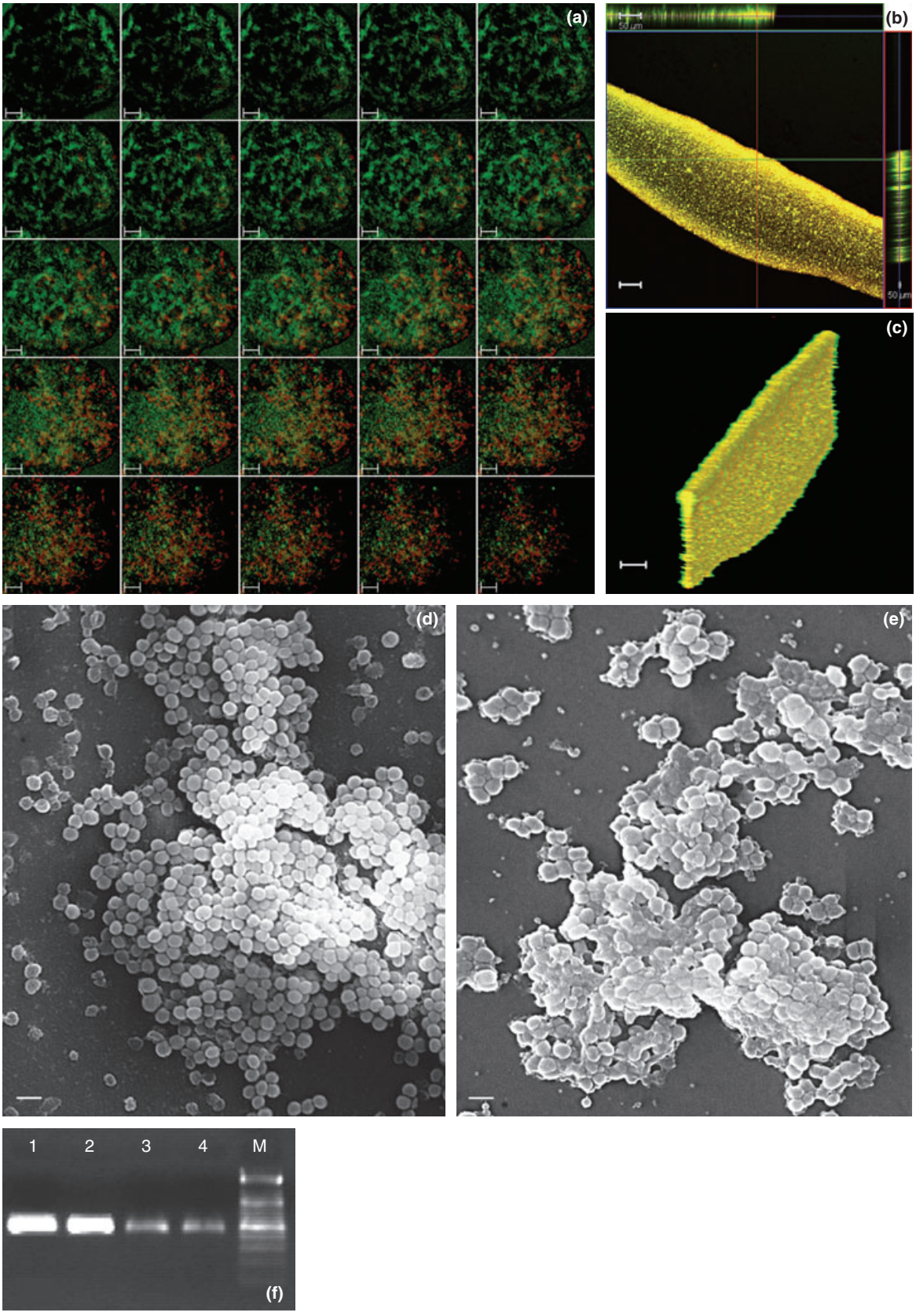
Results

The panel shown in Fig. 1 displays the genotypic status of the environmental *H. pylori* MDC1 strain. The mosaicism in *vac*A alleles was expressed by the s1m1 allelic combination, which revealed by clear bands at 259 and 507 bp for s1 and m1 variants, respectively. The s1m1 *vac*A *H. pylori* MDC1 profile was compared with the *vac*A s2m2 combination of a clinical *H. pylori* strain (*H. pylori* 37/2) in the insert of Fig. 1. The evaluation of the number and type of EPIYA motifs within the *cag*A 3′ variable region revealed the presence of P1P2P3P3 pattern with the repetition of the P3 motif; and fragments corresponding to the *ice*A1 and *bab*A2 genotypes were also detected. The ability of *H. pylori* MDC1 to form biofilm on polystyrene surface was monitored in Fig. 2.

The environmental *H. pylori* studied in this work displayed, after 1 day of incubation without shaking, the presence of clustered cells with mixed coccoid- and rod-shaped cells arranged in a matrix (not shown) similar to that studied in clinical *H. pylori* strains, which were analysed following the same experimental conditions (Cellini *et al.* 2005b).

The noninvasive CSLM technique enabled the visualization of section images of a 2 day mushroom-shaped structure characterized by a thickness of 6·3 μm (Fig. 2a). The apical cap sections (from 1 to 10 section of Fig. 2a) were characterized by green cells, whereas proceeding towards the stalk of the mushroom-type formation, red fluorescence was detected in particular in the external part of the stalk. Regular, parallel water channelling was detected in this structured bacterial population (Fig. 2a). Optical sections were obtained with a 0·3 μm increment on the *z*-axis.

Figure 2 Phenotypic aspects of *Helicobacter pylori* MDC1 environmental strain. Confocal micrograph images showing live/dead staining of *H. pylori* MDC1 biofilm formation at various time intervals (a, b, c). (a) Horizontal optical section in a 2-day-old live/dead stained biofilm. The images show large clamps of living cells (green fluorescent) with images of dead bacteria (red fluorescent) located principally to periphery. The red bacteria increase on the bottom slide. Optical sections were obtained with a 6·3 μm increment on the *z*-axis. Original magnification: 10×. Scale bar: 5 μm. (b) Orthogonal projection gallery of biofilm 20 days old. By confocal scanning laser microscopy (CSLM) investigations, we observe the distribution and the viability of bacteria. Cross-sections display the depth of biofilm. Original magnification: 40×, Scale bar: 50 μm. (c) Rotated view provides a global biofilm prospective. Original magnification: 40×. Scale bar: 50 μm. (d, e) Scanning electron micrographs of aged sessile culture of H. *pylori* MDC1 at 20 days (d) and 30 (e) days with coccoid cells aggregated in a mature biofilm. Original magnification: 10·000×, Scale bar: 2 μm. (f) Agarose gel electrophoresis of *H. pylori* MDC1 reverse transcriptase–polymerase chain reaction (RT–PCR) of *lux*S gene performed at the beginning of the experiments (lane 1), at 2 days (lane 2), 20 days (lane 3) and 30 days (lane 4). M indicates the DNA molecular weight marker (0·1 kbp). The results presented here are representative of those obtained from three independent experiments with two replicates.



Results indicated in Fig. 2b,c displayed sagittal projection of the mature biofilm at 20 days having a thickness of 62·1 μm with a complex and well-organized structure evident with three-dimensional reconstruction, resulting from the compilation of a series of individual *x*, *y* section taken across the *z*-axis.

By SEM observations, from 2 days of incubation, the spherical morphology was prevalent among the total cells, and the sessile cultures were characterized by well-organized large aggregates (not shown) which persisted over time. In particular, the coccoid morphotype was recorded at 20 days (Fig. 2d) and this cellular aggregated community was progressively embedded in an abundant matrix as shown in Fig. 2e for 30 days.

Contrary to the clinical *H. pylori* strains behaviour, aged *H. pylori* MDC1 biofilms resulted well organized with intact coccoid cells (Figs. 2d,e). In particular, in 30 days, *H. pylori* MDC1 biofilm (Fig. 2e), spherical intact cells were surrounded by a conspicuous matrix with an evident presence of blebs, which are shown in Fig. 3.

The expression of the *lux*S gene, related to bacterial quorum-sensing detected in RT–PCR and showed in Fig. 2f, gave positive results in each checked control time. In particular, the *lux*S gene displayed the highest levels of expression till 2 days of incubation and a significant decrease in the subsequent observations (20 and 30 days).

Discussion

Helicobacter pylori is a highly versatile bacterium, characterized by a unique, extraordinary variability that involves the genotype (Cellini *et al.* 2006; Suerbaum and Josenhans 2007), which can be easily revealed by its different behaviour acquired during the cellular cycle and is aimed at the environmental adaptation (Cole *et al.* 2004; Cellini *et al.* 2005b; Azevedo *et al.* 2007).

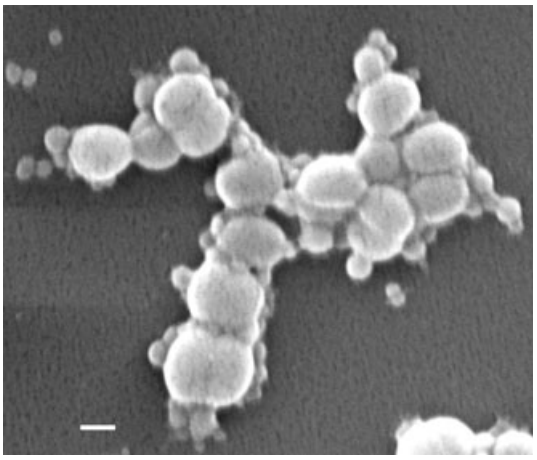


Figure 3 Vesicle production (blebs) from clustered coccoid cells of *Helicobacter pylori* MDC1 at 30 days. Original magnification: 20·000×, Scale bar: 0·5 μm.

In this work, the genotypic and phenotypic characteristics of a marine isolate of *H. pylori* were analysed. The data which were obtained displayed in this micro-organism the presence of significant virulence factors, strongly related to the bacterial pathogenesis. *Helicobacter pylori* MDC1 displayed a *vac*A s1m1 profile related to a great level of vacuolating toxin (Atherton *et al.* 1995), the presence of the *ice*A1 allelic variant which is frequently associated with the presence of peptic ulcer and increases the production of IL-8 in the host (van Doorn *et al.* 1998; Yamaoka *et al.* 1999; Mukhopadhyay *et al.* 2000) and the *bab*A2 positiveness related to Leb binding activity (Appelmelk *et al.* 1997). A particular consideration must be done for the presence of a large number of combination EPIYA phosphorylation motifs harboured in *cag*A gene. In fact, the P1P2P3P3 combination is indicative of a virulence of the strain, because the number of EPIYA motifs results more closely associated with gastric cancer genesis than the detection of *cag*A alone (Rudi *et al.* 1998; Argent *et al.* 2005; Choi *et al.* 2007; Reyes-Leon *et al.* 2007). Thus, the *H. pylori* MDC1 genotypic panel displays the characteristics of a virulent strain which can be potentially pathogenic for the host. On the other hand, it cannot be excluded that such virulence factors might also have a role in the adaptive mechanisms to the environment.

In a previous study, Lu *et al.* isolated and genotyped, for the *vac*A mosaicism, 11 strains of *H. pylori* from samples of untreated wastewater, revealing a prevalence of s1a m1 *vac*A type, suggesting, for faecal contaminated water, a potential role in the transmission of more virulent *H. pylori* strains.

Reguera and Kolter (2005) state that the evolution of virulence in bacteria, such as *Vibrio cholerae*, might be influenced by factors outside the host. In particular, the secretion of toxins could be a potential defence mechanism against environmental predators (Hilbi *et al.* 2007). The environmental isolate characterized in this study, harbours a genotype coding for the most important virulence markers, and in particular, does not lose the cag-PAI, which could exert a role in adapting to the marine environment and which also can be acquired by different species (Datta *et al.* 2003).

Regarding the biofilm formation, *H. pylori* MDC1 displayed a dense and well-structured biofilm, persistent over time, and able to express the quorum-sensing-related *lux*S gene. On the whole, aggregated coccoid *H. pylori* cells, tenaciously attached to the polystyrene surface and well organized in a biofilm, were observed over time.

The phenotypic behaviour of this marine isolate was different with respect to the clinical strains which were analysed under the same experimental conditions (Cellini *et al.* 2005b), unlike the clinical *H. pylori* cultures in which the dispersion of aggregated cells and death occurred after 7 days; structured coccoid *H. pylori* MDC1 cells were observed till 30 days, underlining its acquired ability in persistence over time.

This newly exhibited biofilm persistence observed in the strain isolated in the environment could enable its survival in an ecosystem which demands major challenges towards the external microbiota with respect to the gastric niche. These observations underline the extreme flexibility in the kingdom of the micro-organisms aimed to envision new evolutionary strategies in accordance with the environmental modifications.

The ability of *H. pylori* to form biofilm as an optimal survival strategy can be paralleled with the behaviour of several species in aqueous environments, also including pathogens (Kierek and Watnick 2003; Mai-Prochnow *et al.* 2004).

Therefore, the survival of this virulent strain in a stressing environment, such as seawater, represents a serious risk for the transmission of the bacterium.

The presence of blebs, released from the bacterial surface without loss of membrane integrity, from sessile bacteria population at 30 days of incubation, represents a clear answer to stress stimuli; these particulate features are commonly observed in the matrix of biofilm formed in *Pseudomonas aeruginosa* (Schooling and Beveridge 2006).

The CSLM images, performed for the first time on the *H. pylori* MDC1 biofilm, display a 'mushroom structure' with water channels probably involved in nutrient transport less evident in correspondence of the mushroom stalk, in which some red cells were present. The red fluorescence on the border of stalk could represent an area in which dead cells release protected extra-cellular DNA as a component part of the matrix.

The expression of *lux*S gene found at each time point indicates either the viability of bacterial cells or the cell–cell communication till 30 days. The decreasing level of gene expression observed in aged cultures could represent the reduced need of cross-talking among cells living in a well-structured and already organized community. The evidence of these genotypic and phenotypic *H. pylori* MDC1 characteristics emphasizes the role of the natural environment as potential sources of pathogenic *H. pylori*.

In particular, the micro-organism in the environment adapts itself to the new living conditions through well-known strategies of persistence (Cellini *et al.* 2005a; Oliver 2005; Azevedo *et al.* 2007) guaranteeing a more prolonged protection to the bacterial community. Moreover, this environmental strain combines its stability in biofilm formation to genotypic status corresponding to a strain closely related to gastric pathogenesis.

The persistence of the environmental virulent *H. pylori* strain in a clustered state, such as the biofilm, suggests a long-term survival of the bacterial community outside the host, enabling bacterial transmission with important clinical repercussions.

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References

Adams, B.L., Bates, T.C. and Oliver, J.D. (2003) Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl Environ Microbiol* **69**, 7462–7466.

Ahmed, K.S., Khan, A.A., Ahmed, I., Tiwari, S.K., Habeeb, A., Ahi, J.D., Abid, Z., Ahmed, N. *et al.* (2007) Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Med J* **48**, 543–549.

van Amsterdam, K., van Vliet, A.H., Kusters, J.G. and van der Ende, A. (2006) Of microbe and man: determinants of *Helicobacter pylori*-related diseases. *FEMS Microbiol Rev* **30**, 131–156.

Appelmelk, B.J., Negrini, R., Moran, A.P. and Kuipers, E.J. (1997) Molecular mimicry between *Helicobacter pylori* and the host. *Trends Microbiol* **5**, 70–73.

Argent, R.H., Zhang, Y. and Atherton, J.C. (2005) Simple method for determination of the number of *Helicobacter pylori* CagA variable-region EPIYA tyrosine phosphorylation motifs by PCR. *J Clin Microbiol* **43**, 791–795.

Atherton, J.C., Cao, P., Peek, R.M. Jr, Tummuru, M.K., Blaser, M.J. and Cover, T.L. (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. *J Biol Chem* **270**, 17771–17777.

Azevedo, N.F., Pinto, A.R., Reis, N.M., Vieira, M.J. and Keevil, C.W. (2006) Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed *Helicobacter pylori* to stainless steel 304 and polypropylene. *Appl Environ Microbiol* **72**, 2936–2941.

Azevedo, N.F., Almeida, C., Cerqueira, L., Dias, S., Keevil, C.W. and Vieira, M.J. (2007) Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol* **73**, 3423–3427.

Braganca, S.M., Azevedo, N.F., Simoes, L.C., Keevil, C.V. and Vieira, M.J. (2007) Use of fluorescent in situ hybridisation for the visualisation of *Helicobacter pylori* in real drinking water biofilms. *Water Sci Technol* **55**, 387–393.

Carron, M.A, Tran, V.R., Sugawa, C. and Coticchia, J.M. (2006) Identification of *Helicobacter pylori* biofilms in human gastric mucosa. *J Gastrointest Surg* **10**, 712–717.

Cellini, L., Del Vecchio, A., Di Candia, M., Di Campli, E., Favaro, M. and Donelli, G. (2004) Detection of free and plankton-associated *Helicobacter pylori* in seawater. *J Appl Microbiol* **97**, 285–292.

Cellini, L., Di Campli, E., Grande, R., Di Bartolomeo, S., Prenna, M., Pasquantonio, M.S. and Pane, L. (2005a) Detection of *Helicobacter pylori* associated with zooplankton. *Aquat Microb Ecol* **40**, 115–120.

Cellini, L., Grande, R., Traini, T., Di Campli, E., Di Bartolomeo, S., Di Iorio, D. and Caputi, S. (2005b) Biofilm formation and modulation of *lux*S and *rpo*D expression by *Helicobacter pylori*. *Biofilms* **2**, 1–9.

Cellini, L., Grande, R., Di Campli, E., Di Bartolomeo, S., Capodicasa, S. and Marzio, L. (2006) Analysis of genetic variability, antimicrobial susceptibility and virulence markers in *Helicobacter pylori* identified in Central Italy. *Scand J Gastroenterol* **41**, 280–287.

Chisholm, S.A., Teare, E.L., Patel, B. and Owen, R.J. (2002) Determination of *Helicobacter pylori vac*A allelic types by single-step multiplex PCR. *Lett Appl Microbiol* **35**, 42–46.

Choi, K.D., Kim, N., Lee, D.H., Kim, J.M., Kim, J.S., Jung, H.C. and Song, I.S. (2007) Analysis of the 3′ variable region of the *cagA* gene of *Helicobacter pylori* isolated in Koreans. *Dig Dis Sci* **52**, 960–966.

Cole, S.P., Harwood, J., Lee, R., She, R. and Guiney, D.G. (2004) Characterization of monospecies biofilm formation by *Helicobacter pylori*. *J Bacteriol* **186**, 3124–3132.

Coticchia, J.M., Sugawa, C., Tran, V.R., Gurrola, J., Kowalski, E. and Carron, M.A. (2006) Presence and density of *Helicobacter pylori* biofilms in human gastric mucosa in patients with peptic ulcer disease. *J Gastrointest Surg* **10**, 883–889.

Datta, S., Khan, A., Nandy, R.K., Rehman, M., Sinha, S., Chattopadhyay, S., Das, S.C. and Nair, G.B. (2003) Environmental isolates of *Aeromonas* spp. harboring the *cagA*-like gene of *Helicobacter pylori*. *Appl Environ Microbiol* **69**, 4291–4295.

van Doorn, L.J., Figueiredo, C., Sanna, R., Plaisier, A., Schneeberger, P., de Boer, W. and Quint, W. (1998) Clinical relevance of the *cag*A, *vac*A, and *ice*A status of *Helicobacter pylori*. *Gastroenterology* **115**, 58–66.

Drumm, B. and Sherman, P. (1989) Long-term storage of *Campylobacter pylori*. *J Clin Microbiol* **27**, 1655–1656.

Gerhard, M., Lehn, N., Neumayer, N., Boren, T., Rad, R., Schepp, W., Miehlke, S., Classen, M. *et al.* (1999) Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci USA* **96**, 12778–12783.

Grubel, P., Hoffman, J.S., Chong, F.K., Burstein, N.A., Mepani, C. and Cave, D.R. (1997) Vector potential of houseflies (*Musca domestica*) for *Helicobacter pylori*. *J Clin Microbiol* **35**, 1300–1303.

Hilbi, H., Weber, S.S., Ragaz, C., Nyfeler, Y. and Urwyler, S. (2007) Environmental predators as models for bacterial pathogenesis. *Environ Microbiol* **9**, 563–575.

Joyce, E.A., Bassler, B.L. and Wright, A. (2000) Evidence for a signaling system in *Helicobacter pylori*: detection of a *lux*S-encoded autoinducer. *J Bacteriol* **13**, 3638–3643.

Kabir, S. (2003) Review article: clinic-based testing for *Helicobacter pylori* infection by enzyme immunoassay of faeces, urine and saliva. *Aliment Pharmacol Ther* **17**, 1345–1354.

Kierek, K. and Watnick, P.I. (2003) Environmental determinants of *Vibrio cholerae* biofilm development. *Appl Environ Microbiol* **69**, 5079–5088.

Lu, Y., Redlinger, T.E., Avitia, R., Galindo, A. and Goodman, K. (2002) Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. *Appl Environ Microbiol* **68**, 1436–1439.

Mai-Prochnow, A., Evans, F., Dalisay-Saludes, D., Stelzer, S., Egan, S., James, S., Webb, J.S. and Kjelleberg, S. (2004) Biofilm development and cell death in the marine bacterium *Pseudoalteromonas tunicata*. *Appl Environ Microbiol* **70**, 3232–3238.

Mazari-Hiriart, M., Lopez-Vidal, Y., Castillo-Rojas, G., Ponce de Leon, S. and Cravioto, A. (2001) *Helicobacter pylori* and other enteric bacteria in freshwater environments in Mexico City. *Arch Med Res* **32**, 458–467.

Mizushima, T., Sugiyama, T., Komatsu, Y., Ishizuka, J., Kato, M. and Asaka, M. (2001) Clinical relevance of the babA2 genotype of *Helicobacter pylori* in Japanese clinical isolates. *J Clin Microbiol* **39**, 2463–2465.

Moss, J.A., Nocker, A., Lepo, J.E. and Snyder, R.A. (2006) Stability and change in estuarine biofilm bacterial community diversity. *Appl Environ Microbiol* **72**, 5679–5688.

Mukhopadhyay, A.K., Kersulyte, D., Jeong, J.Y., Datta, S., Ito, Y., Chowdhury, A., Chowdhury, S., Santra, A. *et al.* (2000) Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. *J Bacteriol* **182**, 3219–3227.

Oliver, J.D. (2005) The viable but nonculturable state in bacteria. *J Microbiol* **43**, 93–100.

Park, S.R., Mackay, W.G. and Reid, D.C. (2001) *Helicobacter* sp. recovered from drinking water biofilm sampled from a water distribution system. *Water Res* **35**, 1624–1626.

Parsonnet, J., Shmuely, H. and Haggerty, T. (1999) Faecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *JAMA* **15**, 2240–2245.

Reguera, G. and Kolter, R. (2005) Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. *J Bacteriol* **187**, 3551–3555.

Reyes-Leon, A., Atherton, J.C., Argent, R.H., Puente, J.L. and Torres, J. (2007) Heterogeneity in the activity of Mexican

Helicobacter pylori strains in gastric epithelial cells and its association with diversity in the *cagA* gene. *Infect Immun* **75**, 3445–3454.

Rudi, J., Kolb, C., Maiwald, M., Kuck, D., Sieg, A., Galle, P.R. and Stremmel, W. (1998) Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *J Clin Microbiol* **36**, 944–948.

Sasaki, K., Tajiri, Y., Sata, M., Fujii, Y., Matsubara, F., Zhao, M., Shimizu, S., Toyonaga, A. *et al.* (1999) *Helicobacter pylori* in the natural environment. *Scan J Infect Dis* **31**, 275–279.

Schauder, S., Shokat, K., Surette, M.G. and Bassler, B.L. (2001) The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**, 463–476.

Schooling, S.R. and Beveridge, T.J. (2006) Membrane vesicles: an overlooked component of the matrices of biofilms. *J Bacteriol* **188**, 5945–5957.

Stark, R.M., Gerwig, G.J., Pitman, R.S., Potts, L.F., Williams, N.A., Greenman, J., Weinzweig, I.P., Hirst, T.R. *et al.* (1999) Biofilm formation by *Helicobacter pylori*. *Lett Appl Microbiol* **2**, 121–126.

Suerbaum, S. and Josenhans, C. (2007) *Helicobacter pylori* evolution and phenotypic diversification in a changing host. *Nat Rev Microbiol* **5**, 441–452.

Suerbaum, S. and Michetti, P. (2002) *Helicobacter pylori* infection. *Engl J Med* **347**, 1175–1186.

Yamaoka, Y., Kodama, T., Gutierrez, O., Kim, J.G., Kashima, K. and Graham, D.Y. (1999) Relationship between *Helicobacter pylori* iceA, cagA, and vacA status and clinical outcome: studies in four different countries. *J Clin Microbiol* **37**, 2274–2279.