Characterisation of a *Helicobacter pylori* phage (HP1)

EVELYN HEINTSCHEL VON HEINEGG, H. P. NALIK and E. N. SCHMID

Institute for Medical Microbiology, University of Essen, Hufelandstr. 55, 4300 Essen 1, Germany

Summary. The infection of two *Helicobacter pylori* strains with a phage-containing supernate of the lysogenic *H. pylori* strain IMMi 290/89 resulted in a lytic cycle and propagation of phage HP1. In negatively-stained preparations, the empty phage heads measured 55–60 nm in diameter and mature heads measured 50 nm. The flexible, striated phage tail was c. 170 nm in length and 9.5 nm in diameter. The phage showed a mean density of 1.40 g/cm³ in sucrose-density gradients and contained double-stranded DNA c. 22 000 bp in length.

Introduction

The presence of intracellular bacteriophage-like particles in *Helicobacter pylori* present in biopsy specimens was first reported by Marshall *et al.*¹ and Goodwin *et al.*,² but has since received only limited attention. More recently the isolation of an *H. pylori* strain (SchReck 290) that spontaneously produced bacteriophages *in vitro* was described by Schmid *et al.*³ The object of this study was the characterisation of the *H. pylori* phage HP1 recovered from *H. pylori* strain SchReck 290, including its propagation and isolation, lytic cycle, morphology and genome composition.

Materials and methods

H. pylori strains

The *H. pylori* strain IMMi 290/89 designated SchReck was reported previously to contain bacteriophages.³ The strain was stored in brain-heart infusion broth containing glycerol 15% and sheep blood 20% for 1 year at –80 °C.

Ten different clinical isolates of *H. pylori* (IMMi 733/89, IMMi 23/90, IMMi 31/90, IMMi 41/90, IMMi 42/90, IMMi 71/90, IMMi 75/90, IMMi 187/90, IMMi 188/90, IMMi 189/90) were chosen as indicator strains for the propagation of the phage. The strains were isolated from gastric biopsies from patients attending the Department of Gastroenterology of the University of Essen. The isolates showed the morphology typical of *H. pylori* and rapid urease, catalase and oxidase reactions.

Growth conditions

For permanent subculture the bacteria were grown on Sheep Blood Agar (SBA; CM 55, Oxoid), con-

taining whole sheep blood (Oxoid) 10%.⁴ The plaque assay and the induction procedures were performed on SBA and on IsoSensitest Agar (Oxoid) supplemented with bovine serum albumin (Sigma, No. A 2153) 5% (IBA). The broth cultures were performed with Brain-Heart Infusion Broth (BHIB; CM 225, Oxoid) supplemented with calf serum 10% (FKS; Serva 47900, Heidelberg, Germany) and yeast extract (Flow 30-000-49, Meckenheim, Germany) 0.25% in 5-ml volumes.

All incubation was done at 37°C in GasPak-jars (BBL, Cockeysville, MD, USA) in a micro-aerophilic atmosphere (O₂ 5–7% v/v, CO₂ 8–10% v/v, N₂ 83–87% v/v) (Anaerocult C, Merck, Germany).

Propagation and isolation of the phage

After cultivation on SBA the 10 *H. pylori* strains were harvested and suspended in BHIB to a density corresponding to McFarland turbidity standard No. 1. A volume of 100 µl of the thawed and filtered phage-containing supernate of the *H. pylori* strain IMMi 290/89 was added to the bacterial suspension.

After incubation for 3 days at 37°C the turbidity of the test samples was measured with a spectrophotometer (1101 M, Eppendorf, Germany) at 578 nm and compared with the uninfected controls; 200-µl volumes of the suspensions were streaked out on sheep blood agar to estimate the decrease in viable count and to ensure purity of the remaining bacteria.

The broth cultures with lysed bacteria were centrifuged at 11 000 g (JA 20.1 rotor, J2-21 centrifuge, Beckman, Palo Alto, USA) for 10 min to remove the bacterial debris. The phages in the supernate were sedimented through layers of sucrose 20% and 10% at 284 000 g for 2 h according to McNaughton and Matthews⁵ in a Beckman ultracentrifuge, and re-suspended in 500 µl of PBS. Volumes of 2 ml were layered on to 10.5 ml of pre-formed sucrose 5–60% gradients prepared in PBS. The gradients were centrifuged in a Beckman SW 40 Ti rotor for 4 h at 250 000 g at 5 °C. Twenty 600-µl gradient fractions were col-

Figs. 1a and b. Transmission electronmicrograph of thin sections of agar plaques from *H. pylori* strain IMM42/90 infected with phage HP1 isolated from broth cultures of infected *H. pylori* strain IMM42/90; → indicates submembrane complex typically found in *H. pylori* cells, ►, > indicate mature and empty phage heads, respectively. Bar = (a) 200 nm, (b) 500 nm.

Fig. 2. Transmission electronmicrograph of negatively stained preparations of phage HP1 isolated from broth cultures of phage-infected *H. pylori* strain IMM42/90. Bar = 100 nm.

lected. The sucrose concentration of each fraction was determined with an Abbe-refractometer (Zeiss, Oberkochen, Germany). All steps of the phage isolation were monitored by electronmicroscopy.

Plaque assay

The plaque assay was performed by the single layer method.⁶ After cultivation for 3 days on SBA the 10 *H. pylori* strains were harvested and suspended in 5 ml of

NaCl 0.9% to a density corresponding to MacFarland turbidity standard No. 10. One-ml volumes were poured on SBA and IBA and cultured at 37 °C in a micro-aerophilic atmosphere for 24 h. Volumes of 5 µl of the phage-containing sucrose-gradient-fractions of the infected *H. pylori* strain IMM42/90, and 5 µl of PBS as a negative control, were spotted on the bacterial lawns and cultured for up to 72 h. Examination of plates for plaques was done at 24-h intervals.

Induction methods

Eight *H. pylori* strains (IMMi 733/89, IMMi 23/90, IMMi 31/90, IMMi 71/90, IMMi 75/90, IMMi 187/90, IMMi 188/90, IMMi 189/90) were not susceptible to lysis by HP1 and, therefore, were suspect for lysogeny. Before exposure to induction procedures bacteria were harvested from 3-day old cultures on SBA and suspended in 10 ml of NaCl 0.9% to a density corresponding to MacFarland turbidity standard No. 10. For the mitomycin C assay and the ultraviolet irradiation, 1-ml volumes of the bacterial suspensions were streaked out on SBA and IBA and cultured for 24 h in a micro-aerophilic atmosphere. The mitomycin C assay was performed by placing disks of mitomycin C (1 µg) on the lawns of the *H. pylori* strains.⁷ Ultraviolet irradiation of the bacteria was done with an ultraviolet lamp (254 nm grid tube, 50 W input-power, VI-50C, Bioblock Scientific, Illkirch, France) for 30 s, 60 s, 90 s and 120 s at a distance of 10 cm. Induction by high temperature was performed by pre-incubating the bacterial suspensions in a water bath at 50 °C for up to 10 min; 0.5-ml volumes were poured on SBA and IBA every minute. The bacteria were incubated for up to 72 h and monitored for plaques at 24-h intervals.

Electronmicroscopy

For negative staining with uranyl acetate 2%, samples were taken from the uncentrifuged broth cultures of *H. pylori* strains IMMi 41/90 and IMMi 42/90 and the fractions after sucrose gradient centrifugation.

For thin sections, small squares of the plaques in the lawn of *H. pylori* strain IMMi 42/90 were immersed in 0.2 M cacodylate buffer containing glutaraldehyde 2%. After pre-fixation for 2 h at 4 °C, the specimens were fixed in osmium tetroxide 1% followed by uranyl acetate 2%. The fixed specimens were dehydrated in acetone 30, 50, 70, 90 and 100% and embedded in glycidether (Epon 812, Roth, Germany). The thin sections were stained with lead citrate 1% and screened with an EM 10 (Zeiss, Oberkochen, Germany).

Preparation of bacteriophage DNA

The phage DNA was isolated from *H. pylori* strain IMMi 41/90 uncentrifuged liquid culture. The bacterial nucleic acids as well as the plasmid DNA were digested with DNAase I (Serva, Heidelberg, Germany) 2 µg/ml and RNAase A (Boehringer, Mannheim, Germany) 4 µg/ml. The phage DNA was prepared according to the method of Maniatis *et al.*⁸ The precipitation of the phage particles was performed in a polyethylene-glycol 10% solution omitting the CsCl gradient step. The nucleic acid of the phage was then treated with RNAase A for a second time to determine its sensitivity and to digest remaining bacterial RNA. The phage DNA was cleaved with the

restriction endonuclease *EcoRI* (Boehringer). After digestion the phage DNA was separated in a non-denaturing gel of agarose 1% with ethidium bromide as staining agent.

To estimate the length of the phage genome, the agarose gel was standardised with DNA-fragments of the plasmid pAT 153,⁹ produced by a *HinfI* and *EcoRI* digest.

Results

As the phage-containing *H. pylori* strain IMMi 290/89 could not be re-cultivated after storage for 1 year at -80 °C, 10 *H. pylori* strains were inoculated with the phage-containing supernate from the stored strain to identify potential host strains. After incubation for 3 days two of the 10 strains, IMMi 41/90 and IMMi 42/90, showed reduced opacity in the test preparations in comparison with

Fig. 3. Restriction endonuclease digest of DNA of phage HP1 isolated from *H. pylori* strain IMMi 41/90. A, *EcoRI* digest; B, undigested HP1 DNA; M1, marker pat 153, *HinfI*; M2, marker pat 153, *EcoRI*; → indicates weakly stained fragment.

control tubes. This was confirmed by subculture on SBA.

After incubation for 3 days on IBA, but not on SBA, a clear zone of lysis appeared in the lawn of *H. pylori* strain IMMi 42/90 at the application points of the phage-containing sucrose-gradient fractions. No plaques were seen in the other *H. pylori* strains tested.

Eight *H. pylori* strains were immune or resistant to the phage HP1 in the broth assay and in the plaque assay. Inducing systems, including mitomycin C, ultraviolet irradiation or high temperature, did not show evidence of lysogeny in these strains.

In the epon-embedded samples of the plaques, phage heads with diameters of *c.* 60 nm were found in infected bacteria (figs. 1a and 1b). In negatively-stained preparations of both infected *H. pylori* strains, empty phage heads had diameters in the range 55–60 nm while mature heads measured *c.* 50 nm. The flexible phage tail was about 170 nm in length and 9.5 nm in diameter (fig. 2).

Phage particles with a density of *c.* 1.40 g/cm³ were found in sucrose-gradient centrifugation.

Treatment of the isolated phage nucleic acid with RNAase A digested the remaining bacterial RNA but did not degrade the phage nucleic acid. However, the phage nucleic acid was digested with class II-restriction endonucleases (*Hae*III, *Hinf*I and *Eco*RI) which digest only double-stranded DNA. Therefore the nucleic acid of the phage HP1 was identified as double-stranded DNA. After cleavage with restriction enzyme *Eco*RI, the separation of the phage DNA in the standardised agarose gel resulted in six detectable fragments (sizes are shown in fig. 3). Addition of the fragment lengths resulted in *c.* 22 000 bp for the whole phage DNA.

Discussion

This report for the first time describes a lytic phage infection in *H. pylori* as a pre-condition for propagation and physical characterisation of the phage HP1. To classify this phage, the physical characteristics found in this study were compared with those of other phages. Phage HP1 showed several similarities to members of the family Syphoviridae which possess heads of *c.* 60 nm in diameter, a long non-contractile tail of *c.* 150 nm in length, a mean density of 1.49 g/cm³ and double-stranded DNA with a length of *c.* 45–50 kb.¹⁰

Our findings were compared with the information given in previous publications on the morphology of *H. pylori* phages. Thus Marshall *et al.*¹ found intracellular bacteriophage-like particles as well as released phages in *H. pylori* in two different gastric specimens. These icosahedral phages were of helical structure, without a tail, and measured *c.* 40 nm in diameter. Some of the phages were adjacent to remnants of lysed bacteria. Goodwin *et al.*² also

described tail-less intracellular bacteriophages of icosahedral structure and with diameters of *c.* 80–90 nm in *H. pylori* in a gastric biopsy. The first in-vitro study with more detailed information about a strain of *H. pylori* harbouring bacteriophage (SchReck, IMMi 290/89) was reported by Schmid *et al.*³ Thin-section electronmicrographs of this *H. pylori* strain revealed bacteria packed with empty and filled phage heads with dimensions of *c.* 70 × 60 nm, as well as lysed bacteria with released phages which possessed tails with a length of 120 nm that were adjacent to the bacterial cell wall. The negatively stained preparations of HP1 presented in this study reveal the same morphology as those described by Schmid *et al.*³ An explanation for the differences in bacteriophage sizes and morphology is that there are three apparently different morphotypes of *H. pylori* phages.

Several attempts have been made to differentiate subtypes of *H. pylori*. The most successful studies on the heterogeneity of *H. pylori* strains have been with restriction endonuclease analysis of chromosomal DNA,^{11,12} ribosomal RNA gene patterns¹³ and PCR-amplified differentiation of the urease structural genes.¹⁴ These methods seem to provide reproducible methods for the identification of subtypes of *H. pylori*, although restriction endonuclease analysis of chromosomal DNA produced only minor differences in DNA patterns and was difficult to interpret.¹² Plasmid analysis as a marker of differentiation of *H. pylori* strains^{15,16} seems to be less suitable, since only 19–50 % of *H. pylori* strains carried plasmids, which may have been responsible for the minor differences in gene patterns.¹²

Another approach, both to distinguish *H. pylori* isolates and to support epidemiological studies, may be the introduction of phage-typing, based on the finding of lytic phages in this species. We found that two of 10 *H. pylori* strains were lysed by phage HP1 in broth culture. Although HP1 was propagated successfully in liquid cultures of the *H. pylori* acceptor strains, a reproducible plaque assay on solid media (SBA and IBA) could not be established for routine practice. This might be due to inappropriate growth conditions or deficiencies in the expression of phage-receptors under the chosen conditions. For example, the use of the single layer assay instead of the generally preferable double-layer assay for testing phage activity and the induction procedures were necessary because *H. pylori* cells aggregated in soft agar and did not grow in confluent lawns. A successful search for further lytic phages and appropriate growth conditions is the minimum requirement for the construction of a phage-typing system, which could be a worthwhile approach for differentiation of *H. pylori* strains and may provide additional support to the currently available methods of genomic subspecies differentiation.

We thank B. Meister, M. Hemmler-Roloff, A. Spies, M. Chlupka and B. Menge for their technical assistance.

References

1. Marshall BJ, Armstrong JA, Francis GJ, Nokes NT, Wee SH. Antibacterial action of bismuth in relation to *Campylobacter pyloridis* colonization and gastritis. *Digestion* 1987; **37** Suppl 2: 16–30.
2. Goodwin CS, Armstrong JA, Peters M. Microbiology of *C. pylori*. In: Blaser MJ (ed) *Campylobacter pylori* in gastritis and peptic ulcer disease. New York, Igaku-Shoin Medical Publishers. 1989: 25–49.
3. Schmid EN, von Recklinghausen G, Ansorg R. Bacteriophages in *Helicobacter* (*Campylobacter*) *pylori*. *J Med Microbiol* 1990; **32**: 101–104.
4. Ansorg R, von Recklinghausen G, Pomarius R, Schmid EN. Evaluation of techniques for isolation, subcultivation, and preservation of *Helicobacter pylori*. *J Clin Microbiol* 1991; **29**: 51–53.
5. McNaughton P, Matthews REF. Sedimentation of small viruses at very low concentrations. *Virology* 1971; **45**: 1–9.
6. Kay D. Methods for studying the infectious properties and multiplication of bacteriophage. In: Norris JR, Ribbons DW (eds) *Methods in microbiology*, vol 7a. London, Academic Press. 1972: 195–197.
7. Bryner JH, Ritchie AE, Foley JW. Techniques for phage typing *Campylobacter jejuni*. In: Newell DG (ed) *Campylobacter*, epidemiology, pathogenesis and biochemistry. Lancaster, MTP Press. 1982: 52–56.
8. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning, a laboratory manual. Cold Spring Harbor NY, Cold Spring Harbor Laboratory. 1982: 76–85.
9. Twigg AJ, Sherratt D. Trans-complementable copy-number mutants of plasmid ColE1. *Nature* 1980; **283**: 216–218.
10. Ackermann H-W. Phagentaxonomie 1990. State and problems. *BIOforum* 1991; **11**: 419–426.
11. Majewski SIH, Goodwin CS. Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. *J Infect Dis* 1988; **157**: 465–471.
12. Oudbier JH, Langenberg W, Rauws EAJ, Bruin-Mosch C. Genotypical variation of *Campylobacter pylori* from gastric mucosa. *J Clin Microbiol* 1990; **28**: 559–565.
13. Owen RJ, Bickley J, Lastovica A, Dunn JP, Borman P, Hunton C. Ribosomal RNA gene patterns of *Helicobacter pylori* from surgical patients with healed and recurrent peptic ulcers. *Epidemiol Infect* 1992; **108**: 39–50.
14. Foxall PA, Hu L-T, Mobley HLT. Use of polymerase chain reaction-amplified *Helicobacter pylori* urease structural genes for differentiation of isolates. *J Clin Microbiol* 1992; **30**: 739–741.
15. Penfold SS, Lastovic AJ, Elish BG. Demonstration of plasmids in *Campylobacter pylori*. *J Infect Dis* 1988; **157**: 850–851.
16. Simor AE, Shames B, Drumm B, Sherman P, Low DE, Penner JL. Typing of *Campylobacter pylori* by bacterial DNA restriction endonuclease analysis and determination of plasmid profile. *J Clin Microbiol* 1990; **28**: 83–86.