The Metastable Associations of Bacteriophages and Erwinia amylovora

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Research Article

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Abstract

Cultivation of bacteria and phages on solid media can serve as a model for studying the interaction of phage and bacterial population under the diffusion-limited conditions, which frequently take place in nature. Here we describe bacteriophages VyarbaL and Hena2, the members of the *Molineuxvirinae* and the *Ounavirinae* subfamilies, respectively, that are able to form the pseudolysogenic associations (PA) with their host *E. amylovora* 1/79Sm on solid media. These PAa were stable through multiple passages. The phenomenon of the PA formation between a bacterial culture and bacteriophages affect the effectiveness of preparations based on virulent bacteriophages.

Introduction

Fire blight is a destructive bacterial disease of fruit trees first described in 1780 in the Hudson Valley, New York [1]. The only causative agent of fire blight is *Erwinia amylovora* (Burrill, 1882) Winslow et al., 1920, a member of the *Erwiniaceae* family (NCBI:txid552). Fire blight is the first plant disease for which a bacterial etiology has been proven [2]. According to the European and Mediterranean Plant Protection Organization (EPPO) A2 list (list of organisms, present on the EPPO territory), *E. amylovora* has the status of a quarantine object as a highly harmful pathogen. *E. amylovora* possesses a number of specific virulence factors allowing it to infect a wide range of plant hosts and determine the severity of the symptoms of *E. amylovora* the fire blight. Among other factors, *E. amylovora* synthesizes exopolysaccharides (EPS) amylovoran, serving as a pathogenicity factor, and levan, a virulence factor [3]. Nowadays, the prevention and control of fire blight is carried out through legislative control, agricultural practices, through the treatment of the plants by spraying of chemical agents: antibiotics (streptomycin, oxytetracycline and gentamicin), copper-containing compounds, growth regulators (calcium prohexadione) and by using the biological control [4]. Biological methods of the fire blight control are of scientific and practical interest representing an option for environmentally friendly intervention. Bacteriophages, bacteria and even yeasts are considered as potential biocontrol agents against *E. amylovora* [5, 6, 7].

The possibility to control *E. amylovora* and the development of fire blight by application of bacteriophages was tested on detached flowers of pear, apple-tree and quince [8, 9, 10]. Bacteriophage treatment was able to reduce symptoms and caused the decrease of the *E. amylovora* loads by several orders of magnitude. However, complete eradication of the viable pathogen could not be achieved. The reason for the insufficient effectiveness of the proposed suspensions of bacteriophages as control agents may be that the natural habitat conditions can differ greatly from the laboratory cultivation conditions. In nature the interactions of bacteria with their specific phages frequently take place in structurally complex environments that limit the diffusion of the viruses, for example, on the surface of plant organs, in biofilms or in the soil [11, 12]. Cultivation of bacteria and phages on solid media can serve as a model for studying the interaction of phage and bacterial population under the diffusion-limited conditions.

Under these conditions the establishment of the co-existence of bacteriophages and their sensitive hosts is more likely compared to the conditions of evenly mixed planktonic cultures. Phage- bacteria associations can remain stable over many passages [13, 14, 15, 16]. This state of the bacteriophage-host system is not associated with the formation of true lysogeny and has other reasons for the presence of the bacteriophage in the culture after repeated passaging. For example, the presence in the bacterial population of both sensitive and resistant cells allows maintainance of the bacteriophage reproduction and prevents complete elimination of the bacteria [17]. The phenomenon of carrier state also sometimes reffered to as “pseudolysogeny” (though the later term may have different meanings) has not been studied enough and requires no less attention in the development of biopreparations based on bacteriophages than the ability of bacteriophages to lysogenize or transduce [18, 19, 20].

Here we describe bacteriophages VyarbaL and Hena2 that are able to form the pseudolysogenic associations (PA) with their host *E. amylovora* 1/79Sm. These PAa were stable through multiple passages on solid media.

Materials And Methods

Bacteria and bacteriophage strains and their cultivation.

The bacterial strain *E. amylovora* 1/79Sm (Germany, Spontaneous streptomycin-resistant mutant of 1/79, Cotoneaster sp., 1979) [21] was from our laboratory collection. The bacteriophages VyarbaL and Hena2 were isolated during this study.

For bacteria and bacteriophages propagation, lysogeny broth (LB) medium (10g tryptone, 10g sodium chloride (NaCl) and 5g yeast extract) or lysogeny broth agar (LA) were used. The liquid bacterial cultures were grown in LB with agitation. Cultivation of bacteria and bacteriophages was performed at 28°C in all the experiments.

Bacteriophage isolation

For bacteriophages isolation we used enrichment cultures with *E. amylovora* 1/79Sm strain. Briefly, a 10 g sample of soil was added to an 100 ml Erlenmeyer flask containing 30 ml of LB medium inoculated by 2% v/v of the overnight bacterial culture. The enrichment culture was incubated overnight without agitation at 28°C, then the culture was cleared by centrifuged (6 000×g 25 min at room temp in a table-top microcentrifuge) to remove bacterial cells, and the supernatant was spotted on the double-layer plates inoculated with the indicator bacterial strain. The plates were incubated overnight at 28^{o}C, the isolated phage plaques were selected and resuspended in LB. Bacteriophages were purified by five sequential single plaque isolations.

Bacteriophage DNA sequencing and analysis

Genomic DNA was isolated from high-titer bacteriophage lysates using phenol-chloroform extraction as described in [22] and sequenced using an Ion Torrent Proton sequencer system (Applied Biosystems, USA) according to the manufacturer's instructions. The primary assembly was performed using Newbler version 2.9, resulting in a single contig for each of the bacteriophages. The coverage was x207 for VyarbaL and x105 for the Hena2 phage.

Bacteriophage genome annotation was performed by Prokka [23] with further manual curation. Functional annotations were performed using NCBI BLASTx searches, tRNA gene prediction was accomplished using tRNAscan-SE v. 2.0 [24] и ARAGORN v1.2.41 [25]. Bacteriophage DNA termini and packaging mechanism were determined using PhageTerm [26]. Genetic and physical genome maps of bacteriophages were generated using Proksee (CGView) [27]. Gene cluster comparison was performed using the clinker [28]. The complete annotated genome sequences of *E. amylovora* bacteriophages VyarbaL and Hena2 have been deposited in GenBank under the accession numbers OM486945.1 and OM522317.1, respectively.

PCR detection of the bacteriophages

For specific amplification of the nucleotide sequence in bacteriophage genomes, PCR was performed using the following primers: **McpFw** GGATTGCACCTTCGGCTAAG, **McpRw** CGTATTGACTCAACCAGCGG (VyarbaL major capsid protein, product of 959bp); **H2mcpFw** GCAAATGGCTCACGTTTCC, **H2mcpRw** GTGAACTTACCGTTGTACTGACG (Hena2 major capsid protein, product of 794bp).

Amplification was performed in C1000 thermocycler (BioRad, USA) using the active precise control mode and the following temperature-time profile: denaturation – 5 min at 95 ºC; 32 cycles - denaturation at 95ºC – 30 sec, annealing at 56ºC for Hena2- or 57ºC for VyarbaL-specific pair of primers – 30 sec, elongation at 72ºC – 1 min, chain completion – 5 min at 72ºC; cooling up to 12ºC.

Electrophoresis of genomic DNA or its fragments was carried out in 1% agarose gel using Tris-acetate (TAE) buffer [29].

Study of the PA formation

Subculturing techniques were used to create the PA. The material from the phage plaques grown on the lawn of the host strain was transferred by sterile toothpicks onto a fresh LA plate. After the overnight incubation, if the bacterial growth was observed, the material was transferred to a new plate. Up to 14 passages of PAs were performed with simultaneous control of the phage production. To do this the material of growing PA was transferred on the freshly inoculated lawn of the indicator bacterial culture *E. amylovora* 1/79Sm. The PA study was conducted in 20 replicates for each phage-host system. Here below the PA replicates are referred to as a "PA". For convenience, PA are assigned numbers from 1 to 20.

After the 5th, 8th, 11th, and 11th passages the PA cultures from the plates were re-suspended in LB, and CFU and PFU counts were determined by plating the appropriate dilutions. Bacteriophages were tittered on a lawn of the original strain of the bacterium *E. amylovora* 1/79Sm. The amount of free phage PFU

was also determined by removing potentially infected cells (infection centers) by centrifugation at 13,000 rpm for 10 min immediately after resuspension of the PA sample in LB.

Screening of sensitivity to the phage of bacterial cells of PA was carried out by the drop method [30]. The controversial susceptibility results were additionally tested by the double agar overlay assay.

LPS profile analysis

Lipopolysaccharide (LPS) electrophoresis was performed according to the protocols described in [31], briefly the bacterial biomass was resuspended in a standard Laemmli sample buffer and treated with proteinase K after which the samples were loaded onto 12% SDS-polyacrilamide gel. Silver staining of LPS after the electrophoresis was carried out according to the protocol [31] adapted for preferential staining of periodate-oxidized polysaccharides.

Results And Discussion

In search for potential fire blight control agents, we isolated *E. amylovora* bacteriophages VyarbaL and Hena2 from natural sources on the territory of Belarus. The VyarbaL phage was isolated from the soil sample collected under a tree of the genus *Salix* (Belarus, Rudensk, 2018). Phage Hena2 was obtained from the soil under apple and pear trees collected in a private courtyard (Belarus, Novogrudok, 2017). Bacteriophages VyarbaL and Hena2 form clearly distinguishable plaques when plated using the double-layer agar technique. VyarbaL forms plaques of 3–3.5 mm in diameter with a well-marked halo; Hena2 plaques were smaller, from 0.5 mm to 2 mm in diameter, with jagged edges. When the drops of the concentrated (10^7 PFU mL^{-1}) phage suspension of VyarbaL or Hena2 were applied onto the lawn of *E. amylovora* 1/79Sm, turbid lysis zones were observed.

Genomic DNA was extracted from DNAse treated lysates of bacteriophages VyarbaL and Hena2, and complete genome sequences were determined. Double-stranded DNA genomes of bacteriophages had different sizes: 44768 b.p. (VyarbaL) and 84251 b.p. (Hena2). Using PhageTerm analysis, the headful mode of packaging PAC was concluded for VyarbaL with a defined terminus only on one strand and approximative redundancy of 298 bp. The Hena2 genome has direct terminal repeats (439 bp) and obvious termini.

In the VyarbaL genome 49 open reading frames (ORF) were predicted; no tRNA genes were found (Fig. 1). The genome of the bacteriophage Hena2 encoded 114 ORFs and 26 tRNAs genes. All the genes of the VyarbaL genome have the same orientation whereas Hena2 genes organized in several clusters with different orientations. GC-content of the VyarbaL genome 49.8%, Hena2–43.5% (Fig. 1).

Bacteriophage genome sequences were queried against the viruses (taxid:10239) nucleotide collection (nr/nt) using blastn (carried out on 06/05/2022). Sequence similarity searches revealed that VyarbaL presents 98.44% (coverage 98%) sequence identity with *Erwinia* phages vB_EamP-S2 (NC_047917.1). The bacteriophage Hena2 genome had 99.42% (coverage 100%) of similarity to the phage vB_EamM-M7

(NC_041978.1). Thus VyarbaL (NCBI:txid2923252) is a podovirus that can be classified in the *Caudoviricetes* class, the *Autographiviridae* family, within the *Molineuxvirinae* subfamily, shares the *Eracentumvirus S2* species with the phage vB_EamP-S2. Bacteriophage Hena2 (NCBI:txid2923253) is a myovirus, classified in the *Caudoviricetes* class, within the *Ounavirinae* subfamily, sharing the *Kolesnikvirus M7* species with vB_EamM-M7 virus.

Global sequence alignment of the sequences of the proteins encoded by these two bacteriophages using clinker (identity threshold 0.3) showed that VyarbaL and Hena2 share only one similar protein - lysin, sharing 35.03% amino acid identity (82% coverage) (Supplementary material). The two bacteriophages have genes with the same function: predicted structural genes coding the major capsid protein and tail fiber protein; - holin and endolysin genes, involved in host lysis by bacteriophages; and the packaging gene synthesizing terminase. The bacteriophages possess DNA polymerase, exonuclease, endonuclease and DNA ligase genes, involved in the replication and metabolism of DNA and RNA. The genomes differ in the presence of genes for specific structural and assembly proteins, that reflect different types of the virion organization. The bacteriophage Hena2 genome also contains a diverse set of putative transferases and oxidoreductases. In both bacteriophages we were able to identify genes responsible for the specific interaction between the phage and the host. The VyarbaL bacteriophage gene encodes the EPS-depolymerase enzyme, which explains the presence of a distinct halo around bacteriophage plaques. Note that known *E. amylovora* bacteriophages differ in the Efficiency of plating on strains with different levels of EPS production [32]. The presence of this enzyme in VyarbaL suggests the possibility of overcoming the barrier of the extracellular matrix for interaction with the cell surface.

The RIIA and RIIB genes in the Hena2 genome are reported to confer to the T4 phage the ability to escape Rex exclusion by a λ lysogen [33].

Given the absence of any identifiable genes associated with the lysogenic life cycle in VyarbaL and Hena2 genomes, the inherence of turbid plaques indicates that some mechanisms allow significat fraction of the potentially sensitive host cell to survive the exposure to high bacteriophage concentration and to give rise to the microcolonies. This effect may allow the phage to form long lasting metastable associations with the host in which a fraction of the cells gets lyzed to maintain the bacteriophage reproduction while the remaining fraction of bacteria is somehow (temporally) protected from the phage attack to enable bacterial growth. To test this hypothesis, we analyzed the ability of these two bacteriophages to form the PA.

The PA of the VyarbaL bacteriophage and the *E. amylovora* 1/79Sm bacterial culture were shown to be stable over multiple passages. After 14th passage the bacteriophage active against the parental host strain was present in 90% of the PAs (Fig. 2, supplementary material). Bacteriophage DNA was detected by PCR analysis for the main capsid protein gene (gMCP) of the phage VyarbaL in 95% of the PAs after 14th passage (Fig. 3).

In contrast to VyarbaL system, all but one (#11) PAs formed with the Hena2 bacteriophage lost the phage by the eights passage. The data are presented in Fig. 4 and supplementary material. However the PA #11

produced the phage till the fourteenth passage and was PCR-positive for the gMCP of Hena2 (Fig. 5).

Simultaneously with performing the passages, we determined the CFU, PFU and free PFU counts at the passages 5, 8, 11 and 14 for three randomly chosen PA from each phage-host system (these were PA ## 2, 14 and 16 for VyarbaL system and ##10, 11 and 20 for Hena2 system). The ratio of the total PFU to CFU counts in three randomly selected PAs of VyarbaL system for a series of passages varied from 10^{-2} to 6 x 10^{-4}. The ratio of PFU of free bacteriophage to CFU for the PA of the VyarbaL system in a number of passages ranged from 10^{-2} to 10^{-4} (Fig. 6).

For the PA of the Hena2 system, the PFU / CFU ratio was higher compared to the VyarbaL system, ranging from 10^{-1} to 10^{0}. However, the PAs # 10 and #20 lost the bacteriophage after the 8th and 7th passages, respectively.

The PA are likely to represent co-evolving systems in which both host and phage genotypes and/or phenotypes may differ from the parental bacterial and viral strains [34, 35]. To determine the features of the components we re-isolated the evolved phages from the last (14th) passage from the PAs ## 14 and 16 of the VyarbaL system and from the only PA (#11) of the Hena2 system retaining the phage by the 14th passage. We also obtained 32 bacterial subclones from each of two VyarbaL PAs and 64 subclones from the Hena2 PA #11. These subclones were spot-tested for the sensitivity towards the corresponding original phages and the evolved phages derived from the same PA. In all the cases the bacteria appeared resistant, although some of the Hena2 PA sublcones showed very turbid inhibition zones with both original and evolved phages. However, when phage dilutions were plated no plaques were visible.

We then cross-tested a subset (n = 14) of the subclones from the PAs with VyarbaL and Hena2 phages for the sensitivity to the non-parental phages (Hena2 and VyarbaL respectively). In all the cases the subclones remain sensitive indicating that the mechanisms of the resistance to these two viruses were different (Fig. 8).

It is known that LPSs have great structural diversity and determine many serotypes of Gram-negative bacteria. Also, in some cases, LPS serve as receptor molecules for bacteriophages [36, 37]. To determine if the O antigen alterations are involved in the observed resistance we determined the LPS profiles of the parental strains and 7 subclones from each of the systems studied (Fig. 9) but no differences were observed.

Conclusion

In this paper, we consider the phenomenon of the formation of associations between a bacterial culture and bacteriophages that affect the effectiveness of preparations based on virulent bacteriophages. The study showed that bacteriophages of the *Autographiviridae* family, the *Molineuxvirinae* subfamily and of the *Ounavirinae* subfamily and the bacterial culture *E. amylovora* 1/79Sm form metastable associations.

It can be assumed that a small proportion of phage-sensitive cells in PAs capable of supporting the phage in PA, as well as rapid elimination of phage-sensitive cells and preferential reproduction of phage-resistant cells in the composition of the PA Hena2 and *E. amylovora* 1/79Sm. However, the mechanism of formation of PAs with different stabilities remains unclear and requires the attention of researchers. It would be interesting to study the ability to develop phage resistance in *E. amylovora* bacterial culture cells, as well as the effect of the extracellular matrix, which in bacteria is represented by EPS amylovoran and levan, and the substrate specificity of phages on the scenario of interaction with host cells. The data obtained are useful both for the design of cross-specific phage cocktails and for understanding the events accompanying the phage and bacteria meeting on the surface of plants.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author Contributions

All authors contributed to the study conception and design, all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors report no relevant financial or non-financial interests to disclose.

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Figures

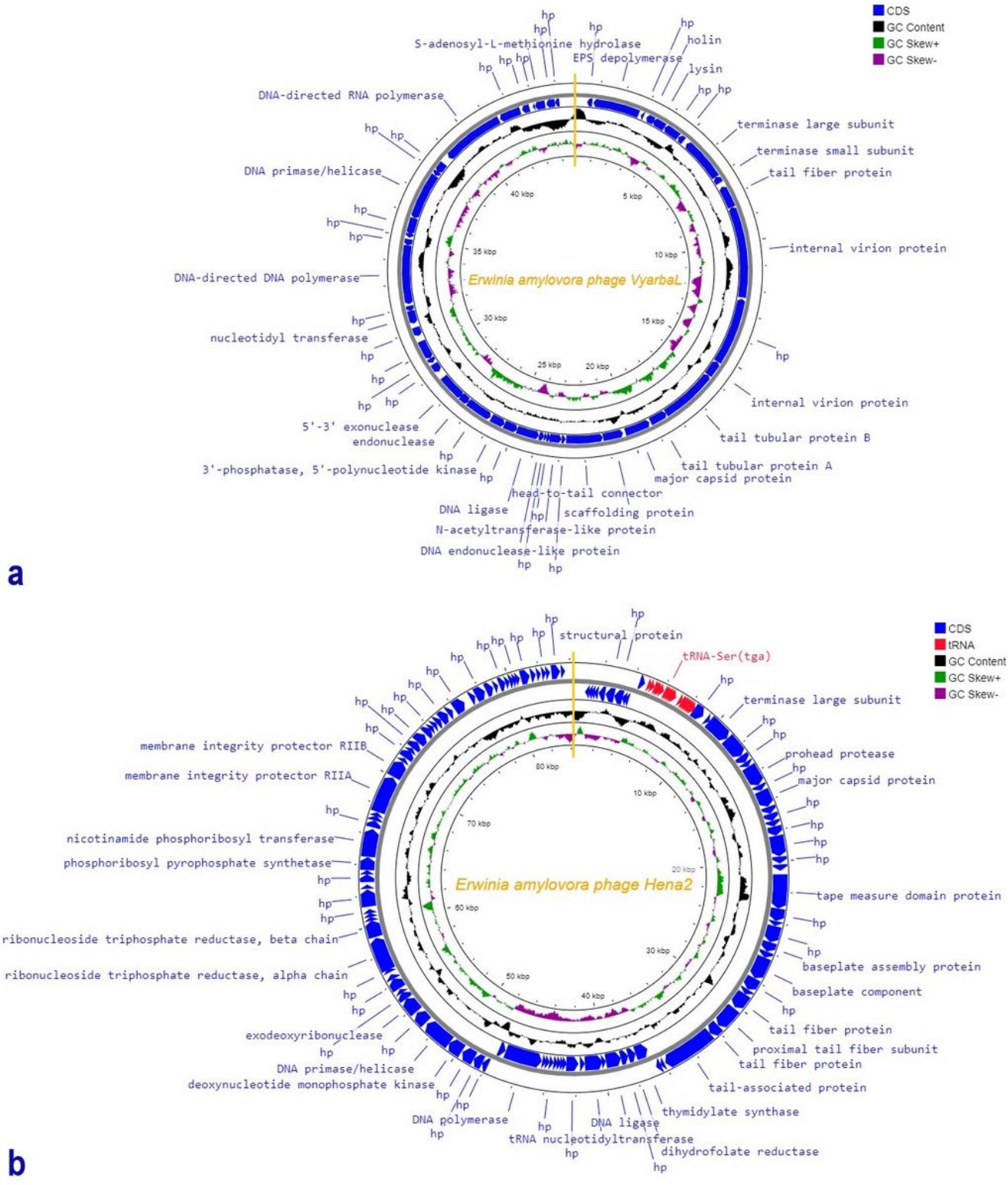

Figure 1

Genomic maps of phages VyarbaL (a) and Hena2 (b) genomes prepared using Proksee

Outer and inner lanes correspond to predicted genes on the forward and reverse strands, respectively (predicted genes are indicated in dark blue, tRNA genes are indicated in red). The two inner circles correspond to the GC plot and the GC skew, respectively. The yellow line depicts the approximate location

of the physical termini of the virion-encapsidated genomes. Circular presentation of the maps was chosen for compaction of the figure.

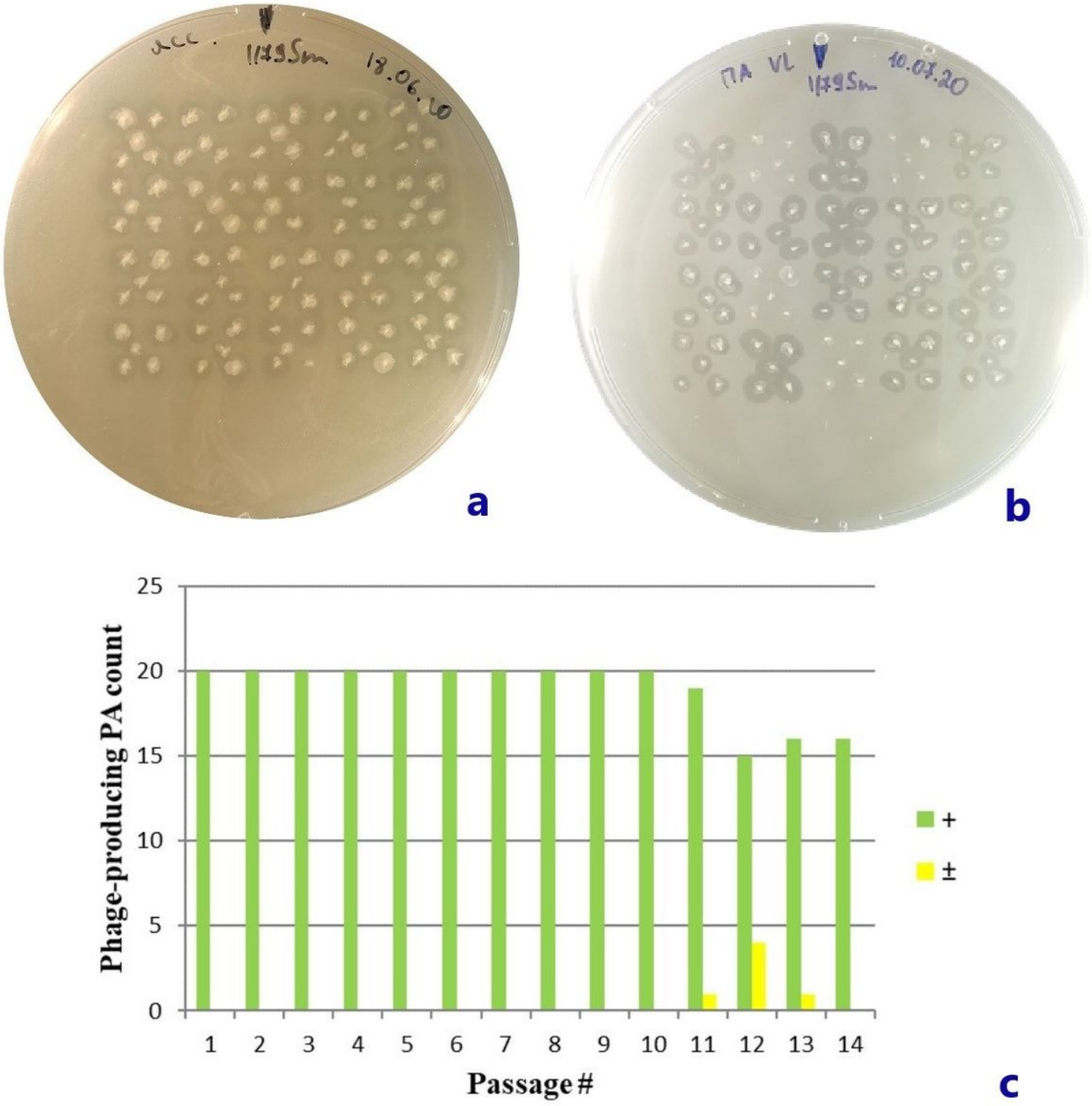

Figure 2

The PA of the VyarbaL and *E. amylovora* 1/79Sm system: a) control of the presence of bacteriophage in passage #5 PA; b) control of the presence of bacteriophage in passage #14 PA; c) bacteriophage in the

14 passages of PAs. In the VyarbaL and *E. amylovora* 1/79Sm system 16 out of 20 PAs retained the ability to produce phage by 14th passage. Note: ± indicates weakly detectable lysis zones.

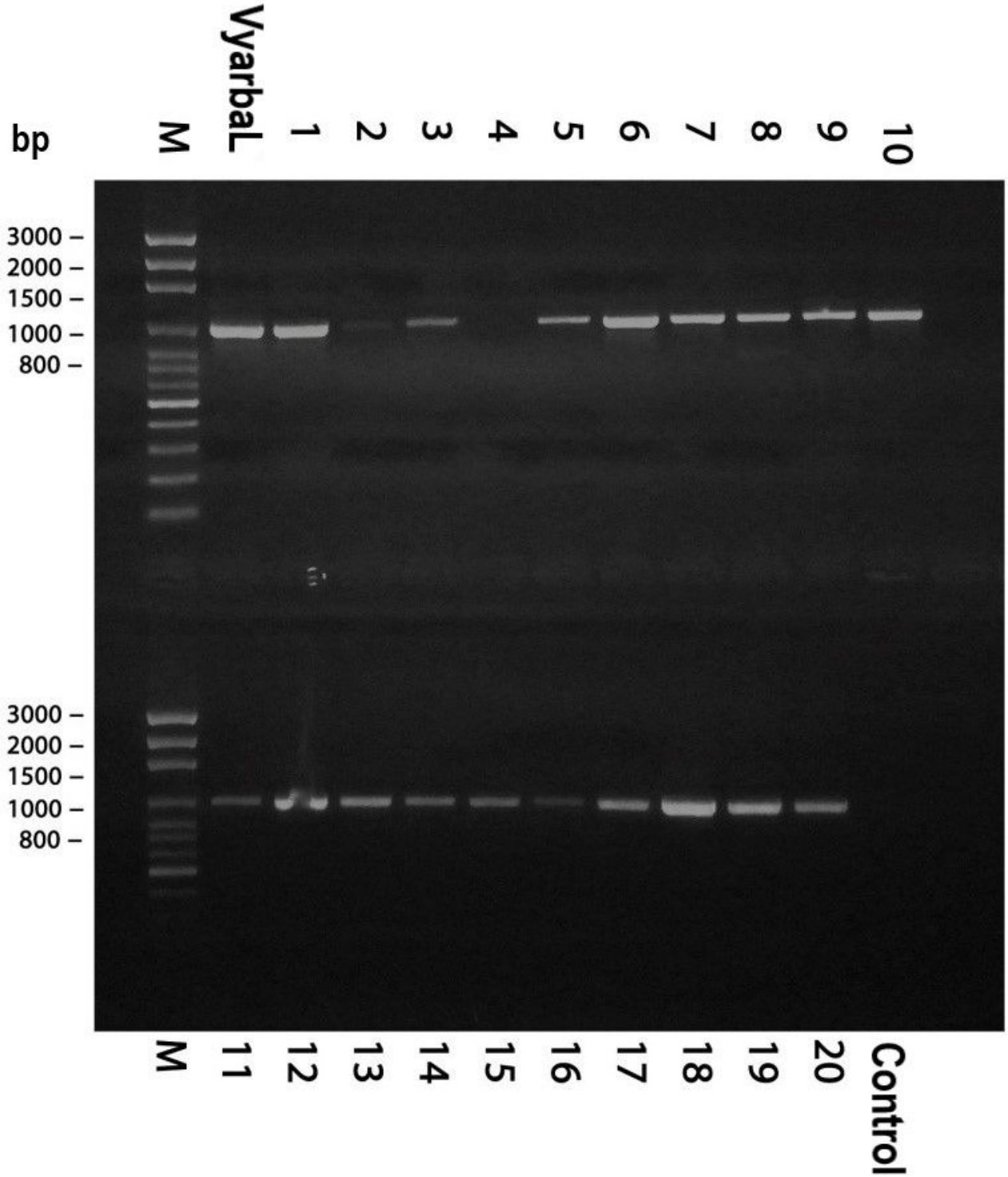

Figure 3

PCR detection of VyarbaL phage DNA in the PA of the VyarbaL and *E. amylovora* 1/79Sm system. 1-20 – PA numbers; M: 100 bp DNA Ladder; VyarbaL lysate – positive control; Control - negative control. Two PA

(## 2 and 4) lost the phage completely that was confirmed by the negative PCR and in another two (## 12 and 18) the lysis was barely detectable on the lawn but PCR reactions were read positive.

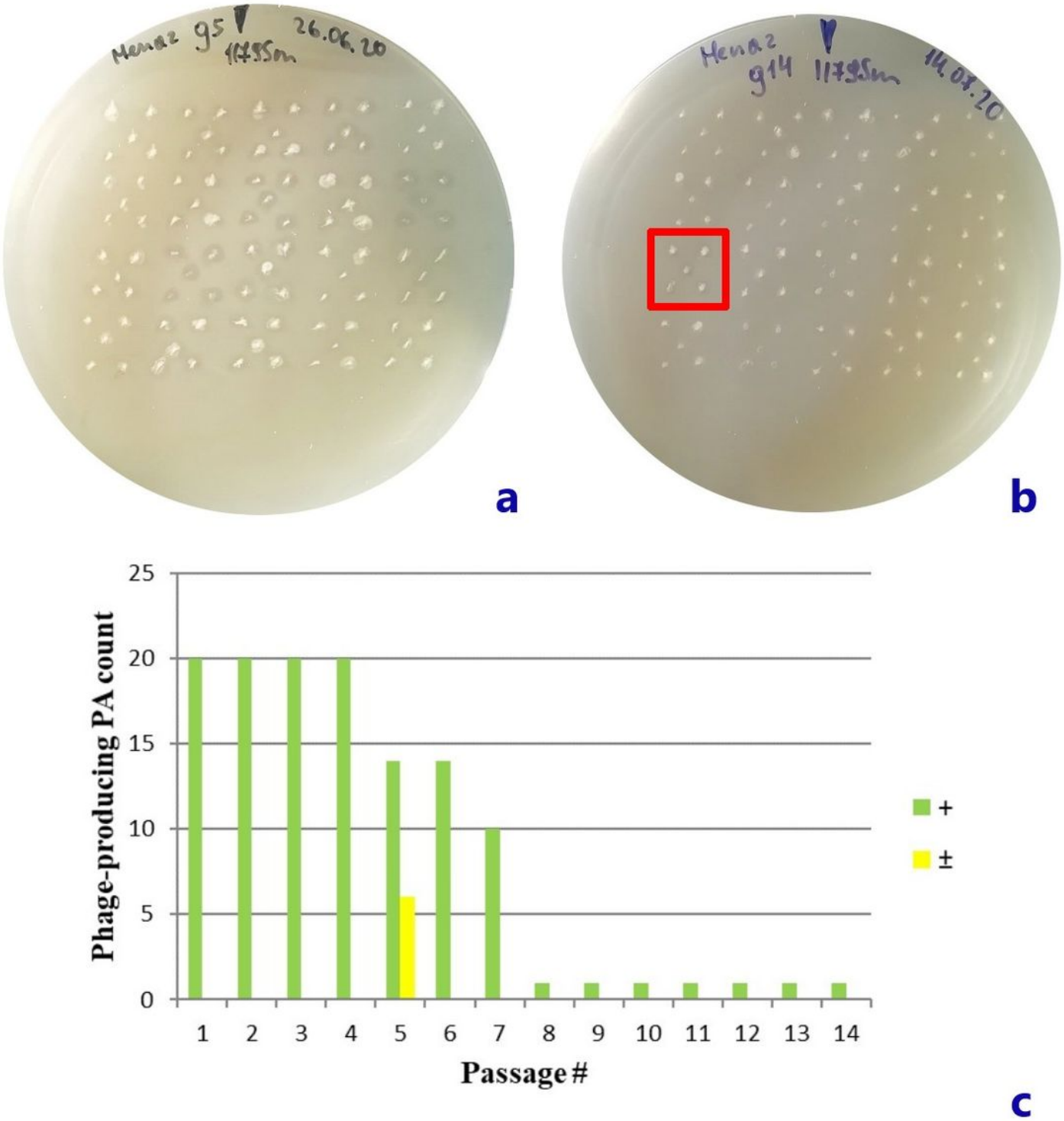

Figure 4

The PA of the Hena2 and *E. amylovora* 1/79Sm system: a) control of the presence of bacteriophage in passage #5 PA; b) control of the presence of bacteriophage in passage #14 PA. The red frame indicates

the phage-producing PA; c) bacteriophage in the 14 passages of PAs. Note: ± indicates weakly detectable lysis zones.

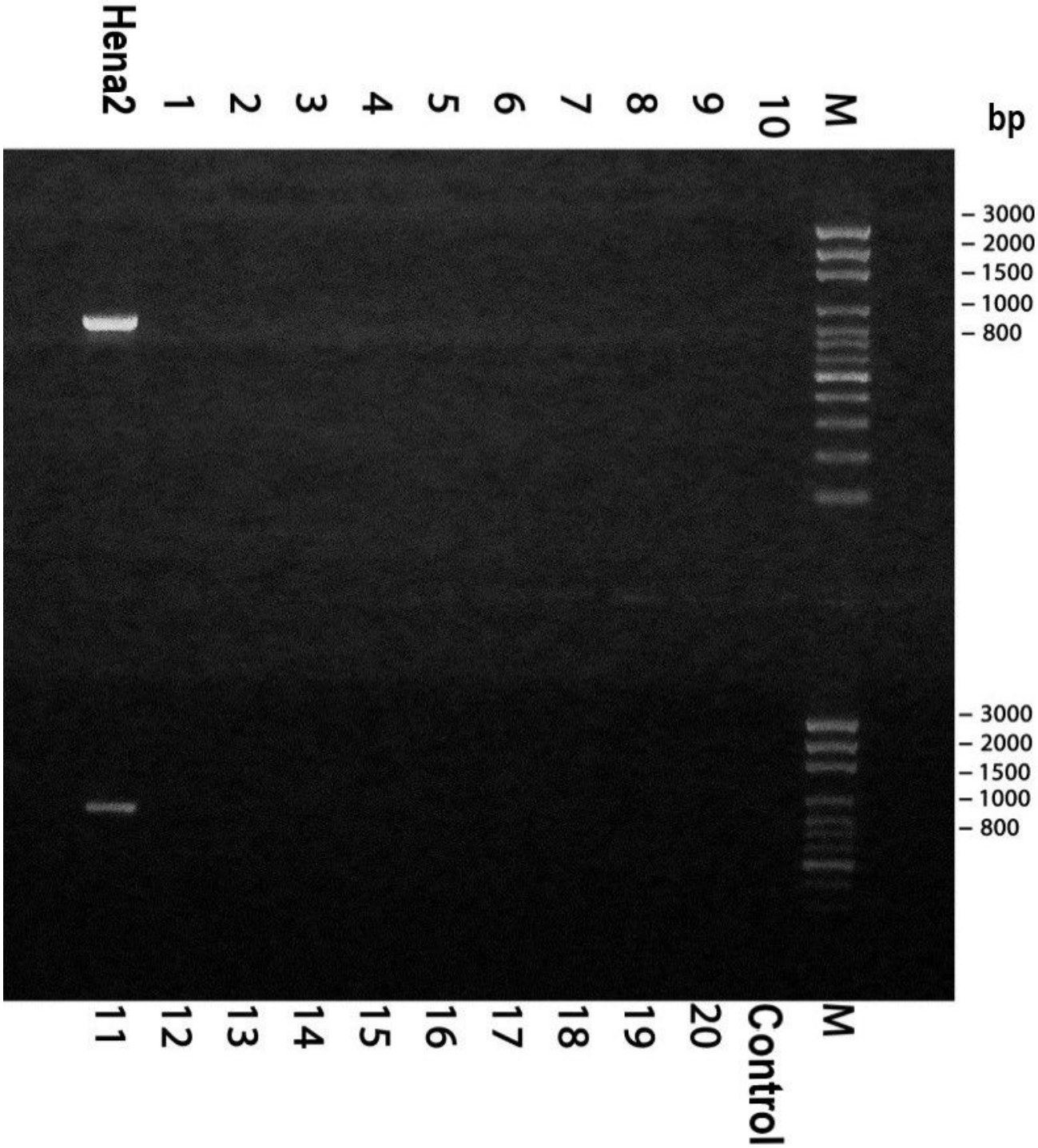

Figure 5

PCR detection of Hena2 phage DNA in the PA of the Hena2 and *E. amylovora* 1/79Sm system. 1-20 – PA numbers; M: 100 bp DNA Ladder; Hena2 – positive control; Control - negative control. Hena2 DNA was

detected using a pair of primers H2mcpFw and H2mcpRw in only one PA out of 20 in passage #14.

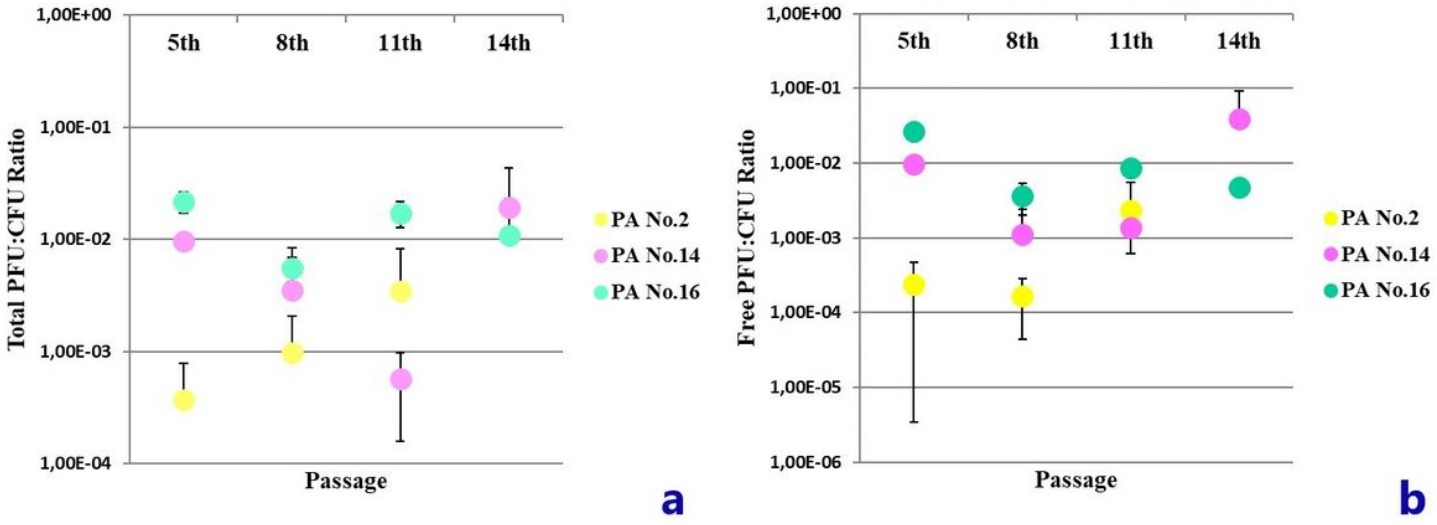

Figure 6

The PFU/CFU ratio (a), as well as the ratio of free phage PFU to CFU (b) in a series of passages for the PA of the VyarbaL and *E. amylovora* 1/79Sm system. The error bars reflect the standard deviation

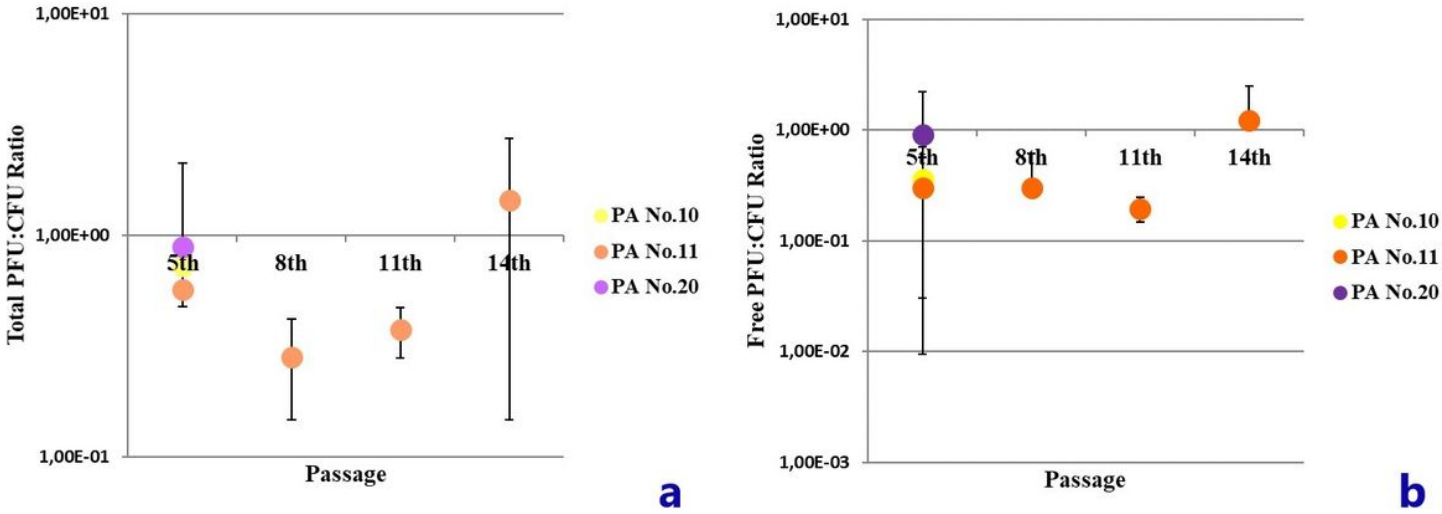

Figure 7

The PFU/CFU ratio (a), as well as the ratio of free phage PFU to CFU (b) in a series of passages for the PA of the Hena2 and *E. amylovora* 1/79Sm system. The error bars reflect standard deviation

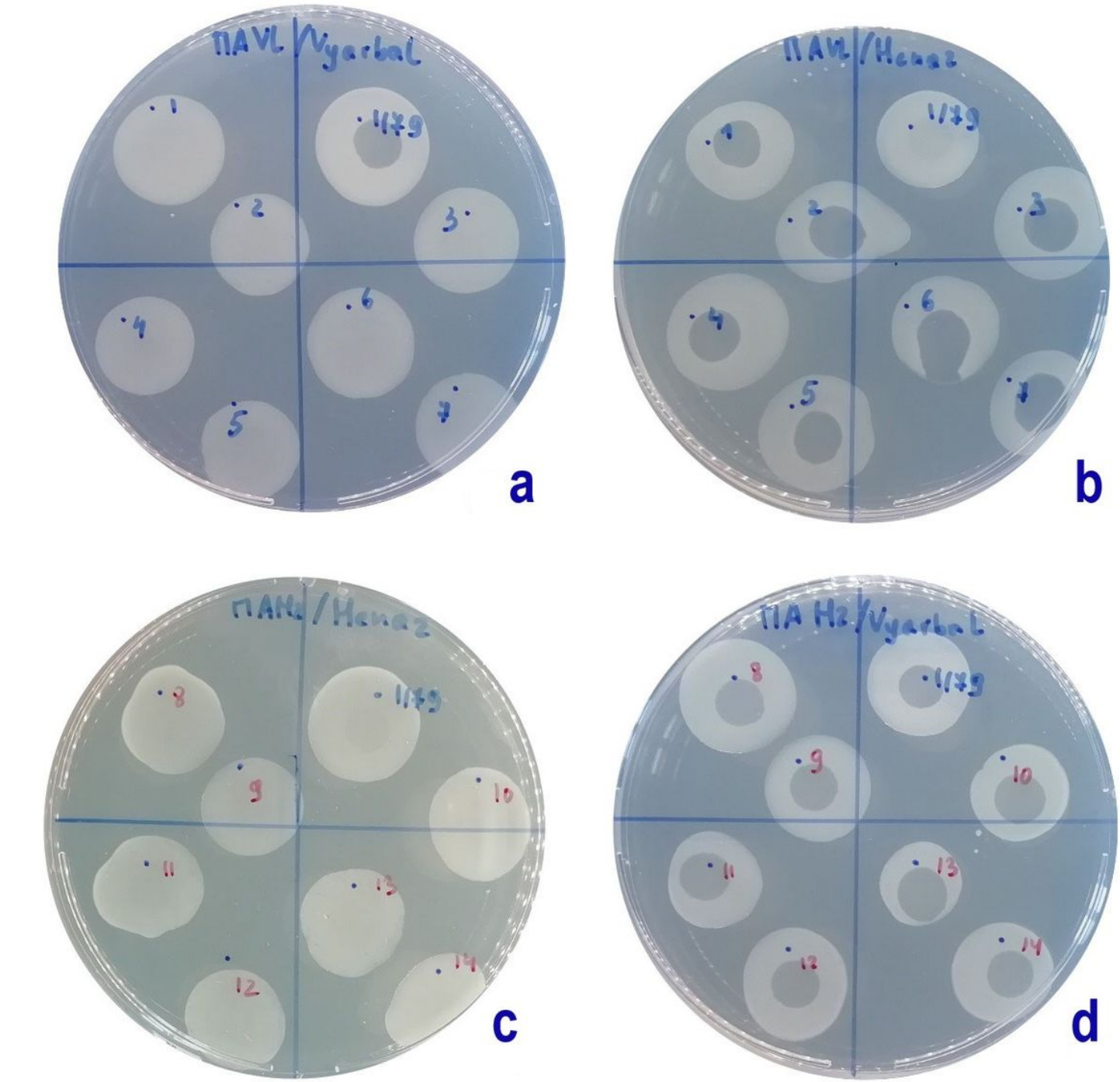

Figure 8

Sensitivity of subclones of the PA system with VyarbaL (a,b) and the PA system with Hena2 (c, d) to stock phages VyarbaL (a,d) and Hena2 (b,c). Note: 1-14: subclone no.; 1/79Sm – stock culture of *E. amyovora* 1/79Sm

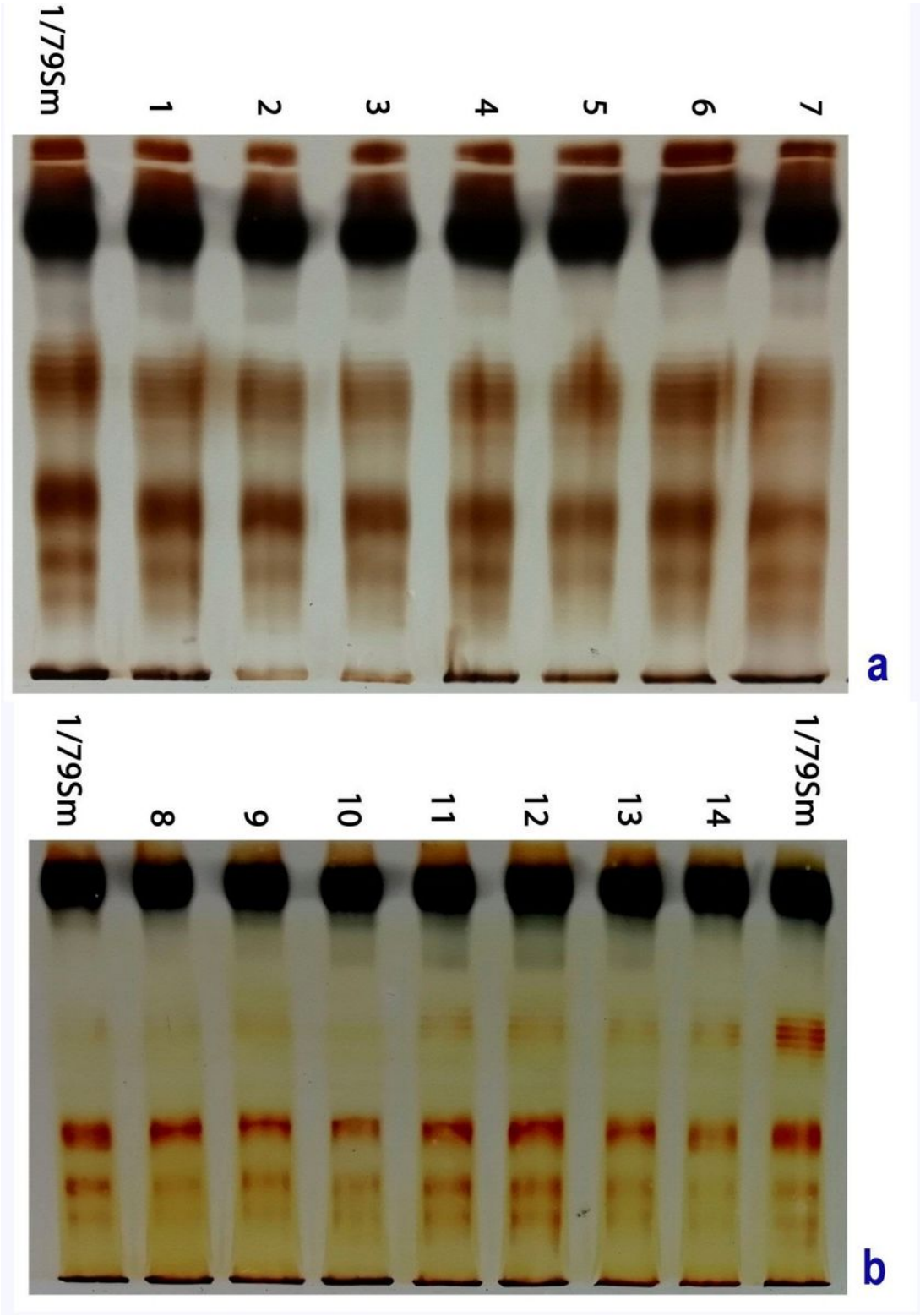

Figure 9

LPS SDS-PAGE of subclones of the PA system with VyarbaL (a) and the PA system with Hena2 (b). Note: 1-14: subclone no.; 1/79Sm – stock culture of *E. amyovora* 1/79Sm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- suppl1.png
- suppl2.docx