

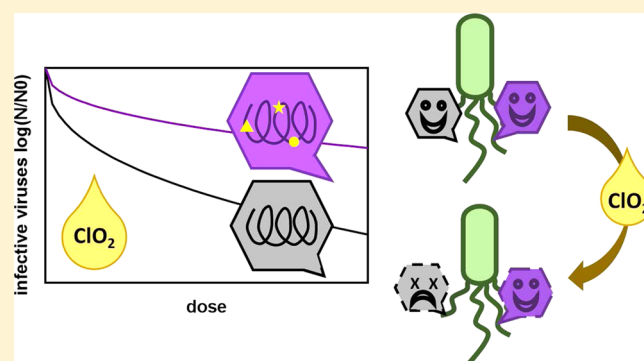
Genetic, Structural, and Phenotypic Properties of MS2 Coliphage with Resistance to ClO₂ Disinfection

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Supporting Information

ABSTRACT: Common water disinfectants like chlorine have been reported to select for resistant viruses, yet little attention has been devoted to characterizing disinfection resistance. Here, we investigated the resistance of MS2 coliphage to inactivation by chlorine dioxide (ClO₂). ClO₂ inactivates MS2 by degrading its structural proteins, thereby disrupting the ability of MS2 to attach to and infect its host. ClO₂-resistant virus populations emerged not only after repeated cycles of ClO₂ disinfection followed by regrowth but also after dilution-regrowth cycles in the absence of ClO₂. The resistant populations exhibited several fixed mutations which caused the substitution of ClO₂-labile by ClO₂-stable amino acids. On a phenotypic level, these mutations resulted in a more stable host binding during inactivation compared to the wild-type, thus resulting in a greater ability to maintain infectivity. This conclusion was supported by cryo-electron microscopy reconstruction of the virus particle, which demonstrated that most structural modification occurred in the putative A protein, an important binding factor. Resistance was specific to the inactivation mechanism of ClO₂ and did not result in significant cross-resistance to genome-damaging disinfectants. Overall, our data indicate that resistant viruses may emerge even in the absence of ClO₂ pressure but that they can be inactivated by other common disinfectants.



INTRODUCTION

Chemical oxidants such as free chlorine (FC) or chlorine dioxide (ClO₂) are widely used to control a number of waterborne pathogens in wastewater and drinking water. However, viruses remain a concern as they are generally more resistant toward disinfectants than traditional bacterial indicators such as *Escherichia coli* and *Enterococci*.^{1,2} In addition, they have very low infectious doses,³ which implies that disinfection must be very effective in order to reduce the risks associated with virally contaminated water.

Compared to other organisms, RNA viruses have high population numbers and high mutation rates that increase the genetic diversity within a population and allow them to rapidly evolve and adapt to environmental stress.^{4–8} It is well-known that resistant viruses readily emerge upon continuous administration of antiviral drugs and, eventually, even among naïve patients that received no clinical treatment.^{9,10} A lesser-known fact is that viruses can also evolve resistance to common water disinfectants. For example, previous studies have demonstrated that poliovirus and F116 bacteriophage developed resistance to chlorine upon repeated exposure to the disinfectant under laboratory conditions.^{11,12} Similarly, it was shown that poliovirus isolated from treated drinking water with

a chlorine residual exhibited greater resistance toward free chlorine as compared to laboratory-adapted strains.¹³ These observations suggest that virus resistance to disinfection has a genetic basis that may be influenced by evolutionary mechanisms.

To date, the adaptation of virus populations to chemical disinfection has not been characterized. This is in part due to the lack of understanding of the specific mechanisms driving virus inactivation upon exposure to disinfectants. Hence, it is difficult to unravel the molecular basis underpinning virus adaptation to disinfection. Such information, however, is important to avoid potential detrimental outcomes of disinfection, such as the selection for viruses that cannot be treated by commonly applied methods. Furthermore, a mechanistic understanding of the resistance mechanisms is important to design alternative treatment strategies for resistant viruses.

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In this study we investigated the emergence of resistance of MS2 bacteriophage to disinfection by chlorine dioxide (ClO₂), and we characterized the underlying mechanisms conferring MS2 resistance to the disinfectant. We chose to work with MS2 because it is a common surrogate for enteric viruses, and because its mechanisms of inactivation by ClO₂ have previously been studied.¹⁴ Specifically, ClO₂ inactivates MS2 by selectively damaging susceptible regions on the structural viral proteins, while the genome remains intact. This results in the inability of MS2 to bind to its host cell and hence in its inactivation.¹⁴ We therefore hypothesize that viruses with resistance to ClO₂ exhibit mutations in their structural proteins that lead to an enhanced stability toward ClO₂ or to alternative binding mechanisms that are less affected by the oxidant. To test this hypothesis, we conducted an experimental evolution assay to obtain ClO₂-resistant MS2 populations. We then identified the mutations fixed in the resistant populations and characterized their effects with respect to the mutants' morphology, their ability to bind to the host, and their aggregation behavior. Finally, we assessed the resistant mutants' replicative fitness and their susceptibility to other inactivating agents, to obtain information regarding the propensity of the disinfection-resistant virus to proliferate in the environment.

■ EXPERIMENTAL SECTION

Briefly, this work is divided into four sets of experiments: first, we conducted experimental evolution studies in order to obtain MS2 population with evolved resistance to ClO₂. Second, we identified the mutations fixed in ClO₂-resistant MS2 populations by genome sequencing. Third, we characterized some phenotypic traits of a subset of ClO₂-resistant populations (host binding, morphology, and tendency to aggregate). Lastly, we investigated the attributes related to the proliferation of resistant MS2, namely the replicative fitness, and the cross-resistance of ClO₂-resistant populations against other means of inactivation (FC, UV₂₅₄, and heat).

MS2 Culturing and Enumeration. Bacteriophage MS2 (DSMZ 13767) and its *Escherichia coli* host (DSMZ 5695) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Propagation and purification of all MS2 populations were conducted as described previously.¹⁵ Infectivity was assessed by enumeration of plaque forming units (pfu) using the double agar layer method.¹⁶ The stock solutions had a concentration of 10¹² pfu/mL and were stored in the refrigerator at 4 °C in phosphate-buffered saline (PBS; 5 mM Na₂HPO₄ (99%, Acros), 10 mM NaCl (99.5%, Acros), pH 7.4).

Chlorine Dioxide Production. A stock solution of ClO₂ was produced by mixing 100 mL of 4% K₂S₂O₈ (99%, Acros) with 100 mL of 2% NaClO₂ (Sigma-Aldrich) and was stored at 4 °C. The resulting ClO₂ concentration (250–1000 mg/L) was determined spectrophotometrically (UV-2550 UV-vis spectrophotometer, Shimadzu) from the absorbance at 358 nm ($\epsilon_{358\text{ nm}} = 1200\text{ M}^{-1}\text{ cm}^{-1}$).¹⁷ The stock solution was diluted with PBS to obtain ClO₂ working solutions with a concentration of 0.5–4 mg/L. Ultrapure water (>18 M Ω cm⁻¹) was used for all aqueous solutions.

Experimental Evolution Assays. In order to obtain resistant MS2 populations, we conducted experimental evolution experiments by subjecting a wild-type (WT) MS2 population to repeated cycles of ClO₂ exposure (Figure S1, SI). Each cycle consisted of treating a virus population (10⁹–10¹⁰ pfu/mL) with ClO₂ such that up to 6 logs of inactivation were

achieved. Following inactivation, the solution with the remaining infective viruses was concentrated using a 100 kDa Microcon centrifugal filter (Millipore, Billerica, MA) and was washed five times with PBS. Finally 50 μ L of solution were collected and spiked into 12 mL of an *E. coli* culture in exponential growth phase at an approximate multiplicity of infection (MOI) of 10⁻⁵ (approximately 10³ pfu viruses per 10⁸ cfu bacteria). Once regrown, the resulting virus populations were purified as detailed previously¹⁵ and then re-exposed to ClO₂. Five replicate experimental evolution assays were conducted of >20 disinfection-regrowth cycles each. The five resulting populations are named E01-E05 hereafter, where E stands for “exposed to ClO₂”. The ClO₂ concentration used was 0.5 mg/L at the beginning of the experimental evolution and was incrementally increased to 4 mg/L in the last cycles.

To test the role of ClO₂ as a selection pressure in the experiments, five control evolution assays were performed in which MS2 populations were only subjected to repeated regrowth but not ClO₂ exposure. The resulting populations are henceforth named NE01-NE05, where NE stands for “non-exposed populations”. Specifically, after each regrowth step, the control populations were diluted, and approximately 10²–10³ pfu were passed to the next cycle. These cycles were repeated 15 (NE01) or 22 times (NE02-05), at which point resistance had emerged.

Kinetic Inactivation Experiments with ClO₂. The emergence of resistance was monitored by comparing the inactivation kinetics of the evolved populations with that of the wild-type after every 5 cycles. Inactivation experiments were carried out in triplicate at room temperature (22 °C) under constant stirring. Specifically, 10 mL beakers were rinsed with 5 mL of ClO₂ working solution before each experiment to eliminate any ClO₂ demand. Then 2 mL of ClO₂ working solution was added to the beaker and was spiked with MS2 stock solution to obtain a starting concentration of approximately 10⁸ pfu/mL. The exact initial virus titer in each experiment was determined by injecting the same volume of MS2 stock solution to a reactor containing 2 mL of ClO₂-free PBS. Initial ClO₂ concentrations ranged from 0.5 to 2 mg/L. To compensate for ClO₂ decay and evaporation during the experiment, a concentrated ClO₂ solution (16 mg/L) was continuously added to the beaker at a flow rate of 10–25 μ L/min using a peristaltic pump (KdScientific). Prior to the start of each experiment it was ensured that this setup was able to maintain a constant desired ClO₂ concentration. Samples (minimum 10 μ L) were taken periodically over the course of 1 to 4 min and were immediately mixed with PBS containing 10% w/v sodium thiosulfate (98%, Sigma-Aldrich) to quench the residual disinfectant. Control samples with sodium thiosulfate added to ClO₂-free virus solution confirmed that sodium thiosulfate did not cause virus inactivation.

Inactivation rate constants for ClO₂ were determined by fitting the experimental data to the Hom model.¹⁸ If the disinfectant concentration is constant during inactivation the Hom model can be expressed as

$$\ln \frac{N}{N_0} = -k_{\text{ClO}_2} C^n t^m \quad (1)$$

where N_0 is the initial infective virus concentration and N is infective virus concentration at time t ; k_{ClO_2} is the Hom rate constant [(mg L⁻¹ min^{m-1})⁻¹]; C is the ClO₂ concentration (mg/L; kept constant during the experiments); m is a model

parameter that describes the deviation from first-order; and n is the coefficient of dilution that attributes the relative importance of time and concentration in the inactivation process. To determine the model coefficients k_{ClO_2} , m , and n , Excel solver and GraphPad Prism were employed to minimize the sum of squares of the difference between the observed and modeled data.

Sanger Sequencing. Single nucleotide polymorphisms (SNP) potentially associated with ClO_2 -resistant MS2 populations were identified by Sanger sequencing as detailed in the [Supporting Information](#).

Host Binding Assays. Binding assays were conducted to test the hypothesis that the host binding mechanism of the resistant mutant was less impacted by ClO_2 than that of the wild-type. Binding of MS2 to their *E. coli* host was determined in triplicate and was quantified as described previously.¹⁹ Briefly, the *E. coli* host was grown to exponential growth phase before being inoculated with MS2 and incubated on ice for 90 min. The samples were then centrifuged, and the bacterial pellet was washed with Tris buffer to remove any free viruses and was then resuspended. The viral RNA was extracted, and quantitative RT-PCR (qRT-PCR) was performed to quantify the number of attached viruses. The qRT-PCR reaction was performed as described previously,¹⁵ using a One Step SYBER PrimeScript RT-PCR Kit (Takara) and a Rotorgene 3000 quantitative PCR platform (Corbett Life Science).

Aggregation. Virus aggregation was assessed by dynamic light scattering and zeta potential measurements. Experimental details are provided in the [Supporting Information](#).

Cryo-Electron Microscopy (Cryo-EM). Cryo-EM was used to reconstruct the structure of the wild-type and mutant viruses, at a 10.5 and 9.5 Å resolution, respectively. The mutant population that most readily yielded good cryo-EM images (NE01) was chosen for this purpose. The relevant experimental and technical details are given in the [Supporting Information](#). Cryo-EM maps of the WT MS2 bacteriophage and its NE01 mutant have been deposited to the Electron Microscopy Data Bank under the accession numbers EMD-8360 and EMD-8396, respectively.

Replicative Fitness. The replicative fitness of the different viral populations was investigated by comparing their growth curves in *E. coli* host cultures. The growth study was performed in duplicate in host *E. coli* strain C3000 (ATCC 15597, LGC Standards, France). This strain was used in order to exclude that the populations only evolved to adapt to the host strain used during the experimental evolution. To conduct the growth experiment, MS2 was added to the host in exponential growth phase at a targeted MOI of 0.01. A low MOI was chosen in order to ensure an excess amount of host cells such that competition among phages for hosts was minimized. The infected culture was incubated during 4–5 h. At intervals of 1 h, sample aliquots were taken, and infective virus titers were enumerated immediately. Additionally, the viral RNA was extracted, and the number of genome copies was measured by qRT-PCR.

In order to quantitatively compare the one-step growth of the populations of interest, the phage growth curve was fitted to the Modified Gompertz model. This model describes the MS2 growth as a succession of lag (eclipse), exponential growth, and asymptote^{20,21}

$$\log\left(\frac{N}{N_0}\right) = A \cdot \exp\left\{-\exp\left[\frac{\mu e}{A}(\lambda - t) + 1\right]\right\} \quad (2)$$

where A represents the asymptote of the growth curve ($\log(N/N_0)$) where N is the concentration of infective viruses or genome copies; μ is the specific growth rate at log phase (hour^{-1}); and λ is the lag period between infection initiation and the time point when phage progeny begin to appear (hours). Model coefficients for the Modified Gompertz model were obtained in GraphPad Prism by minimizing the sum of squares of the difference between the observed and modeled data for each experiment separately. The relative replicative fitness of the different populations was compared by the selection coefficient s ²²

$$s = 1 - \mu/\mu_{\text{WT}} \quad (3)$$

where μ and μ_{WT} are the log phase growth rates of the evolved populations and wild-type, respectively. The evolved population has a superior replicative fitness if $s < 0$.

Inactivation Experiments with Other Inactivating Agents. Inactivation experiments with free chlorine, UV_{254} , and heat were conducted, and the associated inactivation rate constants k_{FC} , k_{UV} , and k_{heat} were determined as described in the [Supporting Information](#).

Statistical Analysis. To determine if the inactivation kinetics or growth rates significantly differed between two or more viral populations, the paired t test analysis or regular one-way ANOVA was applied, respectively, whereby the threshold p -value for statistical significance was 0.05. The goodness-of-fit of the data was evaluated based on the coefficient of determination (R^2), which was determined by GraphPad Prism.

RESULTS AND DISCUSSION

Emergence of ClO_2 -Resistant MS2. In order to promote the emergence of ClO_2 -resistant populations, we subjected five replicates of the same starting (wild-type) population to ClO_2 exposure. Of these, two populations (E01 and E02) exhibited resistance to ClO_2 after 30 and 22 inactivation-regrowth cycles, respectively. Resistance was manifested in significantly slower inactivation kinetics compared to the wild-type population ([Figure 1](#)). Interestingly, as further discussed below, resistance also developed in three of the five nonexposed controls that underwent dilution-regrowth cycles and were thus not exposed to ClO_2 (populations NE01, NE02, and NE03).

As expected from previous studies,²³ ClO_2 inactivation of MS2 deviated from first-order Chick-Watson kinetics ([Figure 1A](#)) but could be well-described by the Hom model ([eq 1](#)). For each individual inactivation experiment, the rate constant k_{ClO_2} and parameters m and n were determined. The m and n values differed by less than 8% and 23% across all experiments, respectively, and variations in m and n over this range changed the associated values of k_{ClO_2} by less than 4%. Consequently, m and n were treated as constant parameters for this study. Their values were determined by simultaneously fitting the Hom model to all the experiments conducted and correspond to 0.396 and 0.756, respectively. The only variable in the Hom model was thus k_{ClO_2} .

Among the five resistant populations, E01 and NE01 demonstrated the highest extent of resistance, as quantified by the relative decrease in k_{ClO_2} compared to k_{ClO_2} of the WT population ($\frac{k_{\text{ClO}_2}}{k_{\text{ClO}_2\text{-WT}}}$; [Figure 1B](#)). While the titer of the WT

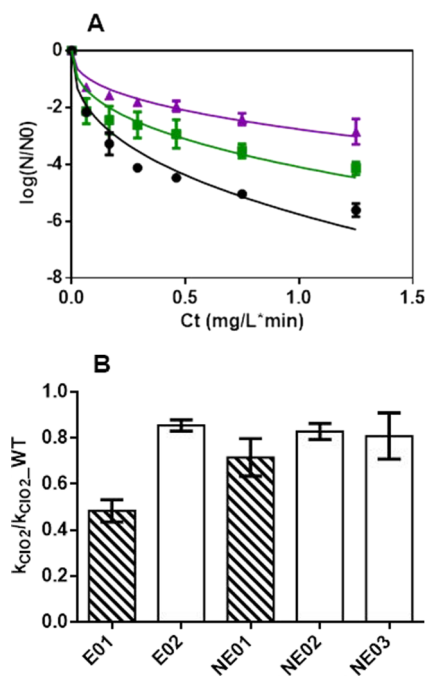


Figure 1. (A) Disinfection kinetics of MS2 upon treatment by ClO_2 . The ClO_2 concentration was maintained at 0.5 mg/L. Black circles: wild-type (WT) population; purple triangles: ClO_2 exposed population (E01); green squares: nonexposed population (NE01). The corresponding fits to the Hom model are shown as solid lines. Error bars represent the range of two replicate experiments. (B) The $\frac{k_{\text{ClO}_2}}{k_{\text{ClO}_2\text{-WT}}}$ of the resistant MS2 populations (two exposed populations E01–02 and three nonexposed populations NE01–03). The ClO_2 concentration was maintained at 0.5 mg/L throughout each experiment. The error bars represent the propagated standard deviation of triplicate experiments. *t* test showed significant differences in the k_{ClO_2} values between WT and the evolved populations at the 95% confidence level: E01 ($p < 0.0001$), E02 (0.01), NE01 (0.002), NE02 (0.007), and NE03 (0.02). Striped bars indicate the two samples that were fully characterized phenotypically, while the other evolved populations were subjected to genome sequencing only.

population decreased by 6 logs at a Ct value of 1 mg/L*min upon exposure to 0.5 mg/L ClO_2 , the infective E01 and NE01 concentration was reduced by less than 3 logs and 4 logs, respectively (Figure 1A). This resistance persisted at higher ClO_2 concentrations: when raising the ClO_2 concentration from 0.5 to 2 mg/L, both E01 and NE01 populations remained more resistant than the wild-type (Figure S2, SI).

While ClO_2 -exposed populations (E populations) developed resistance toward the disinfectant after repeated challenges, the nonexposed samples (NE populations) also became resistant to a similar extent (Figure 1B). From this observation we conclude that ClO_2 exposure was not necessary for the emergence of resistance. Instead, a common pressure present in both exposed and nonexposed samples must have played the dominant role in promoting the emergence of the resistance. Both the exposed and nonexposed virus populations encountered the pressure to efficiently and repeatedly expand the population number under strong bottleneck events. The enhanced ability to proliferate may also benefit the survival under disinfection by ClO_2 . For example, a mutant with a particularly efficient host binding mechanism could both replicate more efficiently and thus dominate regrowth under bottleneck events, as well as better counteract the effects of

ClO_2 on the host binding mechanism. Thus, the effect of ClO_2 exposure as a stressor in the emergence of resistance was seemingly masked by the genetic drift resulting from the frequent regrowth from low virus numbers. A similar conclusion was obtained by Sheldon et al., who reported that the passage of hepatitis C virus in cell culture resulted in viral resistance to drugs.²⁴ It was suggested that the cause of drug resistance was the increased replicative fitness acquired during passage. An alternative explanation for the resistance observed in the nonexposed populations is contamination by exposed viruses. However, the fact that the E and NE populations are not genetically identical (see below) indicates that contamination did not occur.

Genotypic Characterization of ClO_2 Resistant MS2.

The MS2 genome is composed of 3569 nucleotides and encodes for an assembly protein (A protein), a coat protein, a lysis protein, and a replicase. To explore the genetic basis of resistance, the genomes of the WT and the five resistant populations were sequenced. In addition, two evolved populations (E03 and E04) that did not show a significant resistance were included in the analysis. Among these populations, a total of 16 different nucleotide substitutions were identified in the consensus sequences (Table 1). However, the fixation of mutations was only partly reproducible among the different resistant populations, suggesting different resistance pathways. Additionally, the mutation spectrum between exposed and nonexposed resistant populations varied considerably. The exposed populations accumulated more mutations in the A protein (seven, of which five non-synonymous) than the nonexposed ones (three, all non-synonymous), while nonexposed populations possessed more synonymous mutations in the replicase-encoding region (four versus two). Of the mutations incurred in the four exposed populations, seven were also found in one of the nonexposed populations. However, the populations containing the same mutations additionally exhibited divergent ones. These findings, while complex, are consistent with previous literature reports on the genomic content of resistant mutants. In the viral adaptation study of Wichman et al.,²⁵ it was revealed that different amino acid substitutions were identified among experimental replicates, and thus they suggested different trajectories during the adaptation of bacteriophage ϕX174 to high temperature and a novel host. It was also demonstrated that more than one nucleotide substitution can emerge in influenza A virus populations causing resistance to oseltamivir^{26,27} and that therefore biological replicates become equally resistant by the beneficial effect caused by different mutations.^{26,28} Finally, Bordería et al. showed that the adaptation of influenza A virus to a new environment and selection pressure not only occurred by single dominant mutations but also depended on the presence of secondary mutations (minority variants) on the population level. The group contribution of these minority variants could counteract the beneficial effect of the main mutation and induce a fitness decrease compared to the mutation alone.²⁸ Overall, resistance development can thus be a more diverse and complex process than the fixation of a single mutation with the largest beneficial effect in the population.

Table 1 also shows the ranking of the extent of resistance exhibited in the sequenced populations, where 1 indicates the most (E01) and 4 the nonresistant populations (E03 and E04). E01 was more resistant than NE01 by possessing two additional mutations in A protein (Y102F and I238V). This suggests that

Table 1. Mutations Identified in the Virus Populations with Reference to the Genome Sequence of WT^b

position	mutation		population						
	NT	AA	E01	NE01	NE02	E02	NE03	E03	E04
A protein	G155T	R9M	✓	✓					
	C186T	–				○			
	A434T	Y102F	✓						
	A467T	Y113F	✓	✓					
	C513T	–							○
	A841G	I238V	✓						
	A1184G	Q352R						✓	
C1247A	S373Y			✓					
coat protein	T1442G	–				○			
	A1443G	N36D	✓	✓					
	G1656T	A107S					✓	✓	✓
	C1697A	–				○	○	○	○
lysis protein	C1697A	P7Q				✓	✓	✓	✓
replicase	T1946C	–					○	○	○
	T2642C	–					○		
	C2889T	–			○				
	A3239G	–					○	○	○
resistance ranking			1	2	3	3	3	4 ^a	4 ^a

^aNot resistant. ^bThe amino acid position was counted from the initiation of each protein. The ticks and the circles represent nonsynonymous and synonymous mutations, respectively. Nucleotide position 1967 contributes to both the coat protein and the lysis protein region. While it was synonymous in the ORF of coat protein, it caused an amino acid change from proline (P) to glutamine (Q) in the lysis protein.

at least one of these two mutations was directly linked to the increased resistance. NE01, which possessed three mutations, was ranked second in resistance; therefore, at least one of the three mutations exhibited by this population contributed to resistance. Populations E03 and E04, which did not demonstrate significant resistance, nevertheless exhibited six mutations each. Of these, five were shared and were also present in the resistant NE03, which had only one additional (synonymous) mutation. Combined, these results indicate that either mutations exist in E03 and E04 (A1184G and synonymous mutation C513T) that compensated for resistance-inducing mutations or that the synonymous mutation in NE03 can contribute to resistance. The potential role of synonymous mutations is supported by findings by Tubiana et al., who suggested that that synonymous mutations in viral RNA can influence the structure of packaged genome in single stranded RNA viruses, and higher RNA compactness facilitates virus maturation and assembly.²⁹ Therefore, even though MS2 inactivation by ClO₂ does not involve genome damage, synonymous mutations could still influence the virus structure and functions and hence its disinfection susceptibility.

Effect of Mutations on Virus Structure. The majority of the nonsynonymous mutations were located in the structural proteins (A protein and coat protein; Table S1, SI). The only copy of A protein is located near the 5-fold axis of the protein capsid and plays several roles in MS2 life cycle. First, the A protein helps the virus to bind to the *E. coli* F-pilus. Once bound, the retraction of F-pilus pulls the A protein from the capsid leaving a pore such that the viral genome can exit the protein capsid and enter the cell as an A protein-RNA complex.³⁰ The A protein furthermore promotes the virion assembly process by circularizing the viral RNA.^{30–32} The mutations in the A protein may thus influence the binding to the host cells, genome injection, and virus assembly. The

current knowledge of A protein function is still insufficiently detailed to allow us to pin down the significance of each mutation with respect to their influence on viral functions. However, cryo-EM reconstruction of the wild-type and resistant viral particles (NE01) revealed that the biggest structural differences lie in the A protein; the corresponding cryo-EM densities show a Pearson correlation coefficient of 0.84 (Figure 2). Specifically, a more protruding tip can be observed in the A protein of NE01 compared to the wild-type (Figure 2A and C). This result supports our hypothesis that resistance arises from changes in virus motifs associated with host binding.

The MS2 coat proteins serve as the main scaffold of the virus particle, and it encapsulates and protects the viral genome. Upon virion maturation, RNA binds to coat protein dimers and consequently triggers the assembly of the capsid.³¹ Mutations in the coat protein may thus influence virion assembly and structure. Figure S3A (SI) illustrates an MS2 coat protein monomer containing the observed nonsynonymous mutations along with some other interesting residues denoted. Mutation N36D, which was the only mutation present in the coat protein of the two most resistant populations (E01 and NE01; Table 1), is located on the DE loop. While not involved in RNA binding (shown in green in Figure S3A, SI), the DE loop is implicated in the interaction between coat protein dimers to form the full MS2 capsid (Figure S3B and Figure S3C, SI).³³ The substitution of a polar, uncharged asparagine at position 36 by a negatively charged aspartic acid causes a change in the local charge distribution, and this, in turn, could influence the overall protein configuration. Correspondingly, cryo-EM reconstruction revealed a small but measurable difference in the structure of the protein capsid compared to the wild-type (Pearson correlation coefficient of 0.94; Figure 2A), which may influence binding efficiency and specificity.

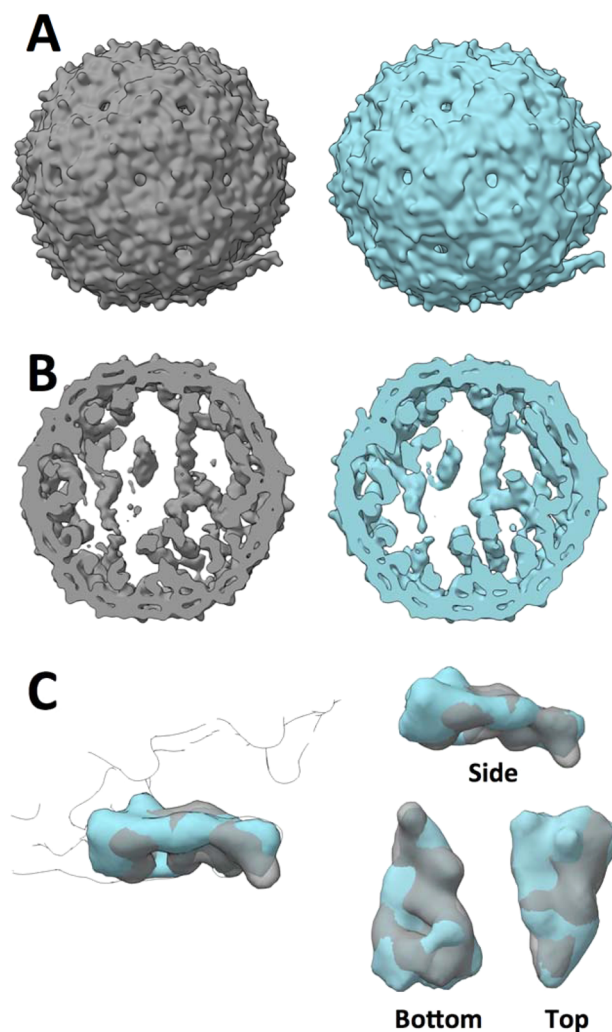


Figure 2. Cryo-EM 3D reconstruction of NE01 (gray) and WT (turquoise) virion particles. (A) The whole virions; (B) Virion particles sliced open exposing RNA organized in single- and double-stranded segments; (C) Superposition of A protein of WT and NE01. The Pearson correlation coefficients for whole virion, coat protein capsid, RNA, and A protein are 0.94, 0.92, 0.89, and 0.84, respectively.

Finally, differences were also found between the RNA structures of the wild-type and NE01 (Pearson correlation coefficient = 0.92; Figure 2B). These differences may influence RNA-coat protein interaction and thus play a part in packaging and assembly efficiency. The RNA structure modification may furthermore influence the stability of the genome and the whole virion.

Resistance Mechanism. To understand how the genetic and structural changes discussed above cause resistance, they must be linked to phenotypic effects. We therefore investigated the most resistant populations with respect to two phenotypic traits that may be linked to resistance, namely the ability to attach to the host cell (E01 only) and the tendency to form aggregates as protection against disinfection (E01 and NE01).

Host Binding. As stated in the Introduction, we hypothesize that resistance of MS2 to ClO₂ is linked to the virus' sustained ability to attach to its host after ClO₂ exposure. To test this hypothesis, the extent of host binding of the wild-type and the resistant populations E01 and NE01 were measured as a function of ClO₂ dose. As shown in Figure 3, the loss in host

binding with increasing ClO₂ exposure was indeed drastically less pronounced for E01 and NE01 compared to WT.

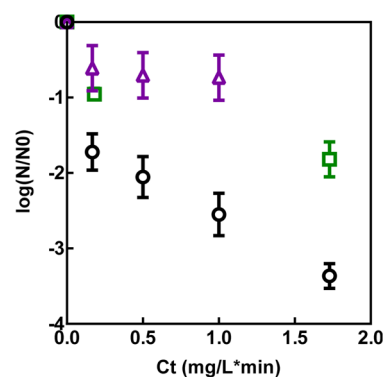


Figure 3. Decrease in host binding as a function of ClO₂ dose for WT, E01, and NE01. Black circles: wild-type population; purple triangles: E01 population; green squares: NE01 population. The error bars represent the standard deviation of triplicate samples.

The more stable host binding of the resistant populations compared to WT is consistent with the differences in genome composition and morphology discussed above. The 3D reconstruction highlighted that the most important structural changes occurred in the A protein, which plays a vital role in the binding of MS2 to its host.³¹ This is consistent with the majority of nonsynonymous mutations in E01 and NE01 being found in the A protein (Table 1). Two of these mutations affected the chemical reactivity of the A protein toward ClO₂. Specifically, it is well-known that ClO₂ preferentially reacts with a subset of amino acids, namely cysteine, tyrosine, tryptophan, histidine, and proline.³⁴ Among the mutations identified on the A protein, two mutations converted ClO₂-reactive tyrosine (Y102 and Y113) to nonreactive phenylalanine. Interestingly, these two tyrosine residues are located in the region of the A protein that is among the most rapidly degraded during exposure to ClO₂.¹⁹ We thus propose that the substitutions to nonreactive residues enhance the chemical integrity of the A protein upon exposure to ClO₂. In addition, the elongation of the A protein structure (Figure 2) may confer physical advantages to binding. Overall, these effects hence allow the resistant populations to better maintain their ability to attach to the host compared to the WT population.

Aggregation. It is known that surface charge modification can lead to a change in the aggregation status of viral particles,³⁵ which in turn can influence the susceptibility of the population toward disinfectants.³⁶ Of the mutations found in E01 and NE01, only one (N36D) leads to a change in charge of the coat protein. This change, however, did not result in enhanced aggregation (see the SI for details). Aggregation could thus be excluded as a cause for enhanced resistance.

Are Resistant Viruses Likely to Proliferate? A fundamental assumption in evolution is the existence of trade-offs;^{37,38} the ability to survive under a given stressor is thus expected to come at the expense of another phenotypic trait. The extent and nature of the specific trade-offs contribute to the likelihood of a mutant to thrive in a given environment. To assess if disinfection-resistant viruses are likely to proliferate, they must be compared with the wild-type with respect to their ability to produce infective progeny and to withstand other stressors, including those typically applied to control viruses. We thus assessed the replicative fitness of resistant populations

E01 and NE01 compared to WT and quantified their cross-resistance to a suite of other inactivating agents.

Replicative Fitness. The replicative fitness, which informs about the virus growth kinetics and final population size, was determined for resistant and wild-type populations in an *E. coli* host (C3000). Growth curves were established for WT, E01, and NE01, to characterize the kinetics of production of both infective viruses and total genome copies (Figure S4, SI). The growth curves were fitted to the Modified Gompertz eq (eq 2). Table S2 (SI) summarized the associated model parameters lag phase, growth rate, and final concentration. The replicative fitness of the different populations was compared by the selection coefficient s for the infective populations (eq 3). For E01 and NE01, positive s values were obtained (0.15 and 0.16), indicating a slightly reduced fitness compared to the WT ($p = 0.04$ and 0.02).

The kinetics of genome replication (Figure S4B, SI) revealed further differences between the populations tested. Specifically, NE01 showed a significantly shorter lag period than WT (by 73%, $p < 0.0001$) and higher asymptote (by 32%, $p < 0.0001$). Despite this greater production of total genome copies compared to the other populations, the output of infectious virus was not higher for NE01. We thus conclude that NE01 had a propensity to produce more defective viral particles during infection.

Cross-Resistance to Other Inactivating Agents. If virus resistance to one disinfectant causes cross-resistance to other disinfectants, their inactivation would become problematic and their proliferation in the environment may pose a serious concern. To assess the cross-resistance of E01 and NE01 to other inactivating treatments, we compared the inactivation kinetics of our resistant and wild-type populations by UV₂₅₄, FC, and heat (Figure 4 and Table S3, SI). While ClO₂ only damages MS2 proteins, UV₂₅₄ mainly targets the genome, where it promotes the formation of pyrimidine dimers and thereby inhibits genome replication. FC causes multiple alterations to both genome and proteins, including protein oxidation, chlorination, carbonylation, and backbone cleavage. Finally, heat causes the denaturation of protein capsid and thus disrupts specific structures needed for host cell binding without inducing chemical modifications.¹⁹ Given the diverse mode of action of these inactivating agents, it is interesting to assess if the resistance observed herein is specific to ClO₂, or if it is also manifested toward other inactivation agents acting by different mechanisms.

For each of the three inactivation agents tested, the inactivation kinetics of the WT and the ClO₂-resistant populations were mostly similar (Figure 4), indicating that ClO₂-resistant viruses remain susceptible to other inactivating treatments. For UV₂₅₄ treatment, the inactivation rate constants of the different virus populations were not significantly different (Table S3, SI). This result was expected, given that UV inhibits genome replication, whereas MS2 resistance to ClO₂ is rooted in the protein-mediated binding function. In contrast, the ClO₂-resistant populations also exhibited a small extent of resistance toward FC, albeit only at a 90% confidence level (Table S3, SI). This may indicate that the enhanced protein stability of the mutants toward ClO₂ has a similar effect on FC. This effect is attenuated, however, because FC is less selective compared to ClO₂ in the targeted protein residues, and because inactivation by FC involves a significant genome component.¹⁹

Finally, inactivation by heat at 72 °C was significantly faster for E01 than for WT, while the difference between NE01 and

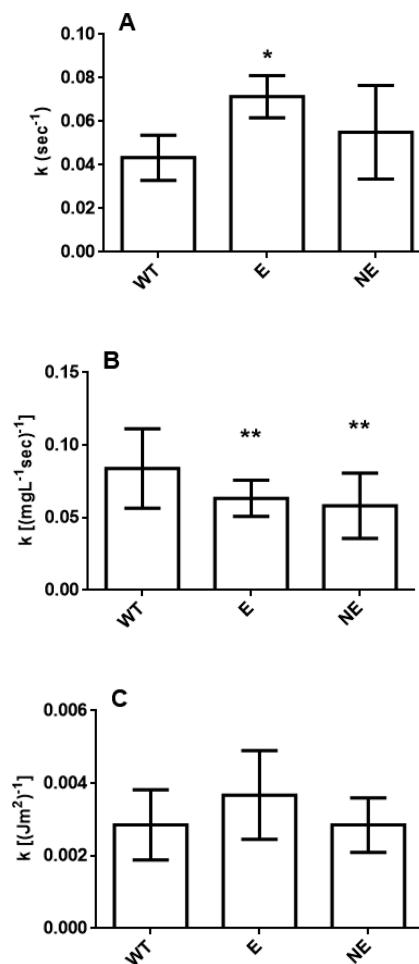


Figure 4. Inactivation rate constants of WT, E01, and NE01 for treatment by heat (A), free chlorine (B), and UV₂₅₄ (C). Rate constants were determined from simultaneous fits of the data to equations S1–S3 (Supporting Information) to duplicate experiments. The error bars represent the associated 95% confidence intervals. Statistically significant differences between rate constants of the evolved populations and the WT are indicated by a single asterisk (*) for the 95% confidence level ($p < 0.05$) and by a double asterisk (**) for the 90% confidence level ($p < 0.1$).

WT was not statistically significant. The more ClO₂-resistant populations E01 and NE01 were thus the least persistent when challenged by heat. Although heat also acts on proteins, the mode of action does not involve oxidation. Hence the oxidation-resistant mutations did not necessarily contribute to the defense against thermal denaturation. Instead, the mutations decreased the thermal stability of the virus particles and made them less thermotolerant.

Given their only slightly impaired replicative fitness, and their susceptibility to inactivating agents that act by a different mechanism than ClO₂, we conclude that ClO₂-resistant viruses may be able to circulate but that they can be controlled by other common water and wastewater disinfection methods.

Implications for Virus Control. This study provides evidence for the development of resistance of a bacteriophage to ClO₂ disinfection. Resistance arose from different mutations and different mechanisms and could emerge simply through repeated regrowth cycles, without the need for ClO₂ exposure. Hence, the mere cycling of a human virus in a population, which involves sequential rounds of excretion and reinfection of

new hosts, may render a virus population increasingly ClO₂ resistant. Furthermore, if the exposure to a disinfectant does accelerate the accumulation of resistance-inducing mutations, then resistant viruses would be most abundant where disinfection is widely used. While these notions remain to be tested, they are supported by the findings that environmental isolates of human viruses can be more resistant to disinfection than the corresponding lab strains¹³ and that some of the resistant strains were isolated from disinfected waters.^{13,39}

Many disinfection systems consist of two sequential stages of disinfection, which sometimes involve different disinfectants.⁴⁰ This approach could be beneficial for the inactivation of resistant viruses, since our work shows little evidence of cross-resistance, in particular if the two disinfectants act by different mechanisms. However, such a setup may also lead to the selection of mutants that exhibit multiple resistances to different disinfectants. Further work is required to determine if such viruses can emerge, if they can be found in the environment, and if they are likely to proliferate.

Finally, it is known that drug-resistant viruses can circulate for years even after the drug pressure was removed.^{41,9,10} Disinfectant-resistant viruses circulating in the environment could thus potentially preserve their resistance over long periods of time. This work contributes to a better understanding of the pressures that promote such resistant viruses and the possibilities to control them.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04170.

Additional experimental information on Sanger sequencing, virus aggregation, Cryo-EM analysis and inactivation by FC, UV₂₅₄, and heat; diagram of experimental evolution setup; the inactivation kinetics of MS2 with different initial ClO₂ concentrations; figures showing MS2 coat protein monomer and dimer interactions at quasi 5-fold and 6-fold axes; one-step growth curve comparison of WT and the mutants and the model fitting parameters; inactivation rate constants in the inactivation experiments using FC, UV₂₅₄, and heat (PDF)

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Notes

The authors declare no competing financial interest.

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