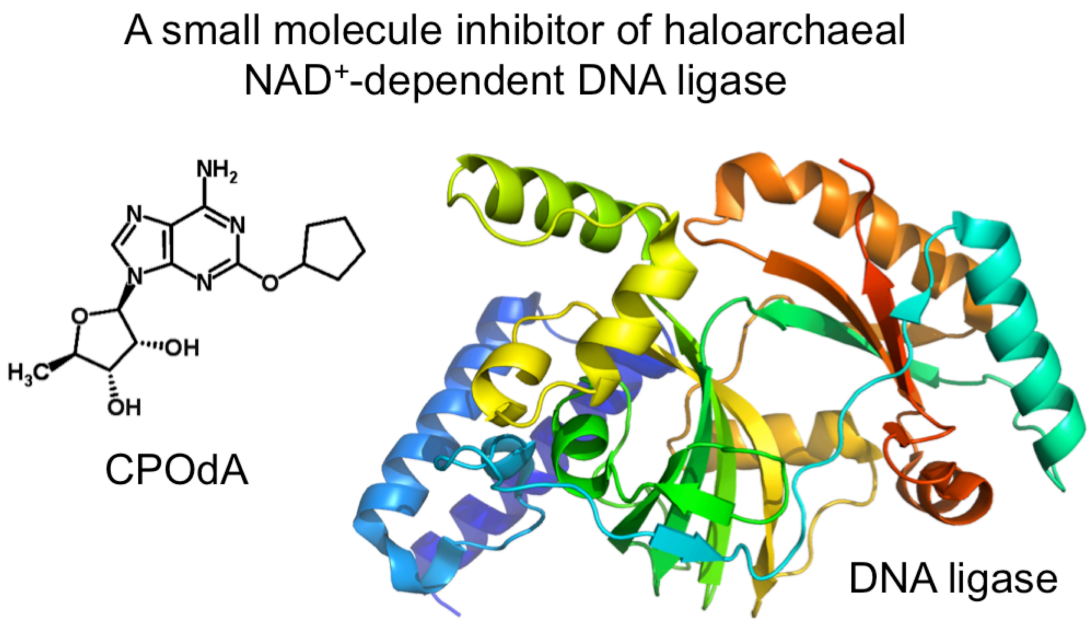

Inhibiting NAD^+-dependent DNA ligase activity with 2-(cyclopentyloxy)-5'-deoxyadenosine (CPOdA) offers a new tool for DNA replication and repair studies in the model archaeon *Haloferax volcanii*

Xavier Giroux and Stuart A. MacNeill

Inhibiting NAD^+-dependent DNA ligase activity with 2-(cyclopentyloxy)-5'-deoxyadenosine (CPOdA) offers a new tool for DNA replication and repair studies in the model archaeon *Haloferax volcanii*

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Running title: Inhibiting *Haloferax volcanii* DNA ligase LigN with CPOdA

Keywords: DNA ligase, *Haloferax volcanii*, archaea, DNA replication, DNA repair.

Abstract

DNA ligases play an essential role in many aspects of DNA metabolism in all three domains of life. The haloarchaeal organism *Haloferax volcanii* encodes both ATP- and NAD^+-dependent DNA ligase enzymes designated LigA and LigN, respectively. Neither LigA nor LigN alone is required for cell viability but they share an essential function, most likely the ligation of Okazaki fragments during chromosome replication. Here we show that 2-(cyclopentyloxy)-5'-deoxyadenosine (referred to as CPOdA), originally developed as a inhibitor of bacterial NAD^+-dependent DNA ligases, is a potent inhibitor of the growth of *Hfx. volcanii* cells expressing LigN alone, causing chromosome fragmentation and cell death, while cells expressing LigA are unaffected. Growth inhibition occurs at significantly lower CPOdA concentrations (MIC ≤ 50 ng/ml) than those required for inhibition of bacterial growth (≥ 2 μg/ml). CPOdA has the potential to become a vital tool in DNA replication and repair studies in this important model organism.

Introduction

DNA ligases play key roles in DNA transactions in all forms of cellular life by catalysing the joining of DNA strands by a mechanism that involves three successive nucleotidyl transfer reactions (reviewed by Tomkinson *et al.*, 2006). Formation of a covalent enzyme-adenylate intermediate (step 1) is followed by transfer of AMP from a co-factor to the 5' phosphate of a DNA strand to form a DNA-adenylate complex (step 2). The ligase enzyme then catalyses attack by the 3' hydroxyl group on the DNA-adenylate complex (step 3), joining the ends of the DNA and realising AMP. DNA ligases enzymes fall into two classes depending on their co-factor specificity: NAD^+-dependent DNA ligases (EC 6.5.1.2) are found predominantly in bacteria, some archaea and a few eukaryotic viruses, whereas ATP-dependent DNA ligases (EC 6.5.1.1) are found predominantly in eukaryotes and archaea, in bacteriophages and in a few bacteria (reviewed by Tomkinson *et al.*, 2006). The major essential role of DNA ligases *in vivo* is the ligation of Okazaki fragments generated during lagging strand DNA synthesis, but DNA ligases also play vital roles in all DNA repair processes and in recombination.

Archaea represent the third domain of life on Earth, comprising at least 20% of the planet's biomass and making a major impact on both biosphere and atmosphere. Current data suggests that eukaryotes may have evolved from within an archaeal lineage (Spang *et al.*, 2015), which would explain the close similarities between archaeal and eukaryotic cellular processes, especially in the area of information processing (DNA replication, transcription and translation). These similarities have led to the archaea being adopted as key model organisms for understanding the fundamental mechanisms of eukaryotic information processing: much of what is known of the detailed structural biology of several key conserved DNA replication factors has come from studies of their archaeal orthologues, for example.

The halophilic euryarchaeon *Haloferax volcanii* has proved to be a powerful model system for genetic analysis of the processes of DNA replication and repair in the archaea (Giroux & MacNeill, 2015). This organism grows aerobically on plates or in liquid at an optimum temperature of 40-45°C in medium containing high levels of salt (routinely 2.5 M NaCl) with a generation time of 2-3 hours and is highly amenable to reverse genetic analysis (Farkas *et al.*, 2013). This has allowed analysis of a number of factors involved in different aspects of DNA replication, repair and recombination including replication origin binding proteins (Norais *et al.*, 2007), the MCM helicase (Kristensen *et al.*, 2014), various single-stranded DNA binding proteins (Stroud *et al.*, 2012, Skowyra & MacNeill, 2012), the XPF/Mus81/FANCM protein Hef and the Holiday junction resolvase Hjc (Lestini *et al.*, 2013, Lestini *et al.*, 2010), and the Rad50-Mre11 complex (Delmas *et al.*, 2013, Delmas *et al.*, 2009). Replication origin function has also been characterised in *Hfx. volcanii* using strains in which single or multiple origins are deleted (Hawkins *et al.*, 2013).

Hfx. volcanii encodes two DNA ligases (Zhao *et al.*, 2006, Poidevin & MacNeill, 2006): an archetypal archaeal ATP-dependent enzyme designated LigA (where A was intended to signify ATP-dependence) and an NAD^+-dependent enzyme designated LigN (with N signifying NAD^+-dependence), the gene for which was most likely acquired by lateral gene transfer (LGT) from a bacterium. Neither LigA nor LigN alone is essential for *Hfx. volcanii* cell viability but deletion of both genes is not possible, indicating that the enzymes share an essential function, presumably Okazaki fragment ligation (Zhao *et al.*, 2006).
The essential nature of NAD^+-dependent DNA ligase activity in bacteria has highlighted these enzymes as potential drug targets and several inhibitors of bacterial DNA ligase activity have been described (for examples, see Mills *et al.*, 2011, Stokes *et al.*, 2011, Stokes *et al.*, 2012, Pergolizzi *et al.*, 2015, Yadav *et al.*, 2015). Here we describe the effects of one such inhibitor, 2-(cyclopentyloxy)-5'-deoxyadenosine (hereafter abbreviated to CPOdA), shown in Fig. 1A, on *Hfx. volcanii* cells expressing one or other or both DNA ligases. We show that cells expressing either the ATP-dependent ligase LigA alone, or both LigA and the NAD^+-dependent enzyme LigN, are insensitive to CPOdA, while cells expressing LigN alone are acutely sensitive to the drug, indicating that LigN is the target of CPOdA action. Inhibition of LigN results in rapid growth arrest, chromosome fragmentation and loss of viability. The efficacy of CPOdA in *Hfx. volcanii* adds a potentially useful tool to the toolbox for analysis of DNA replication and repair processes in this important model system.

MATERIALS AND METHODS

Strains and growth medium

Hfx. volcanii strains used in this study are listed in Table 1. All strains were grown at 45°C in Hv-YPC liquid medium supplemented with 40 µg/ml thymidine and 40 µg/ml hypoxanthine as described in the Halohandbook v7.2 (www.haloarchaea.com/resources/halohandbook). For CPOdA sensitivity assays, cultures were grown to mid-exponential phase (OD_{650nm} of 0.2-0.4) before being serially diluted in 18% SW. Aliquots (5 µl) were then spotted on Hv-YPC plates containing thymidine and hypoxanthine (as above) and varying concentrations of CPOdA and incubated at 45°C for 3-5 days. 2-(cyclopentyloxy)-5'-deoxyadenosine (CPOdA) was obtained from Astra Zeneca's Infection Innovative Medicines Unit (Waltham, MA) as a dry powder, dissolved in DMSO at 20 mM and stored at -20°C.

Pulsed-field gel electrophoresis

CPOdA was added to a final concentration of 0.5 µg/ml to exponentially growing *Hfx. volcanii* cultures in Hv-YPC medium (supplemented with thymidine and hypoxanthine, as above) at 45°C. For PFGE (Kish & DiRuggiero, 2008), samples containing ~ 1 x10^9 cells were taken before CPOdA addition and 30 minutes, 1, 2, 4, 6 and 8 hours after addition, harvested by centrifugation (1800 *g*, 10 minutes), resuspended in 500 µl of 18% SW and mixed with 500 µl of molten agarose mix (2.8 ml 30% SW, 1.2 ml dH_2O, 64 mg agarose, held at 55°C). Plugs were formed using disposable plug molds (BioRad 170-3713) and after 15 minutes at 4°C, ejected into 20 ml of Proteinase K solution (250 mM EDTA pH 8.0, 1% sarcosyl, 1 mg/ml Proteinase K) and incubated at 56°C overnight. Plugs were then washed for 30 minutes at 37°C in 20 ml 1X wash solution (25 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0), incubated overnight at 37°C in 20 ml 1X wash solution containing 0.5 mM PMSF, washed four times for 30 minutes at 4°C with 20 ml wash solution and stored at 4°C in 10 ml 0.1X wash solution until required. Prior to use, in order to generate DNA fragments easily resolved by PFGE, plugs were digested overnight at room temperature (20°C) with 40 units of restriction endonuclease *Swa*I (New England Biolabs R0604) in 1X NEBuffer 3.1 in a total volume of 250 µl. Prior to PFGE, plus were equilibrated for 1 hour in 0.5X TBE. The PFGE samples were then run on 1.2% agarose gels in 0.5X TBE using a BioRad CHEF III apparatus. Run conditions: 6V/cm, 120° angle, initial switch time 0.6 seconds, final switch time 75 seconds, 24 hours, 12°C. Gels were then stained with ethidium bromide (0.1 mg/ml) and bands visualised under UV light. Fragment sizes were determined by comparison with Lambda Ladder PFG markers (New England Biolabs N0340).

RESULTS

CPOdA is a potent inhibitor of *Hfx. volcanii* growth in cells lacking LigA

In order to test the efficacy of CPOdA on *Hfx. volcanii*, wild-type (H53 and H98), Δ*ligA* and Δ*ligN* strains (see Table 1 for full genotypes) were grown to mid-exponential phase in Hv-YPC medium supplemented with thymidine and hypoxanthine (see Materials and methods) and ten-fold serial dilutions spotted onto Hv-YPC plates containing CPOdA at concentrations ranging from 0 to 100 µg/ml. The plates were then incubated at 45°C for 3-5 days. As can be seen in Fig. 1, the growth of wild-type and Δ*ligN Hfx. volcanii* cells on Hv-YPC agar was unaffected by CPOdA at concentrations up to 100 ng/ml, with no reduction in growth rate being seen (Fig. 1B, 1C). In contrast, the growth of Δ*ligA* cells was inhibited by CPOdA at concentrations above 50 ng/ml and abolished altogether at 100 ng/ml (Fig. 1B). These cells are dependent LigN function for viability, as indicated by our previous inability to isolate Δ*ligA* Δ*ligN* double deletion strains by different routes (Zhao *et al.*, 2006), but are otherwise

identical to wild-type. The observed effects (inhibition of cell growth and concomitant loss of cell viability, data not shown) can therefore only be the result of CPOdA inhibition of LigN.

Inhibition of LigN by CPOdA leads to chromosome fragmentation

In order to examine the consequences of LigN inhibition by CPOdA, we analysed chromosome structure using pulsed-field gel electrophoresis (PFGE) in cells treated in liquid culture with the inhibitor. Wild-type (H53, H98), Δ*ligA* and Δ*ligN* cells were grown to mid-exponential phase in Hv-YPC (supplemented with thymidine and hypoxanthine) and CPOdA added to a final concentration of 0.5 μg/ml. Samples were taken for PFGE analysis prior to CPOdA addition and 30 minutes, 1, 2, 4, 6 and 8 hours after addition, and were processed as described in Materials and methods. Prior to PFGE, chromosome plugs were digested with *Swa*I restriction enzyme. This enzyme cuts the main chromosome of *Hfx. volcanii* lab strains in five places, producing fragments of 1.7 Mb, 1.43 Mb, 230 kb, 82 kb and 26 kb (Hawkins *et al.*, 2013, Hartman *et al.*, 2010). *Swa*I also cuts the endogenous plasmid pHV3 at two locations, producing 418 kb and 20 kb fragments. Neither the 26 kb (main chromosome) nor the 20 kb (pHV3) fragment is visible on the PFGE gels. Plasmid pHV1 is not cut by *Swa*I and is also not visible on the gels.

Fig. 2 shows the effects of CPOdA addition to wild-type, Δ*ligA* and Δ*ligN* cells. As expected, addition of CPOdA has no effect on the migration of chromosomes from wild-type (H53, H98) or Δ*ligN* cells. However, addition of CPOdA to Δ*ligA* cultures results in loss of the bands corresponding to the 1.72 Mb and 1.43 Mb chromosomal *Swa*I fragments after 4 hours and marked reduction in the intensity of the 418 kb (pHV3), 230 kb and 82 kb bands. This is accompanied by an increase in smearing in the lower parts of the gel. These results are consistent with CPOdA addition and the resulting inhibition of LigN activity causing fragmentation of the *Hfx. volcanii* genome.

DISCUSSION

DNA ligases are essential enzymes for the joining of DNA in all three domains of life. Most archaeal organisms encode a single ATP-dependent enzyme but many haloarchaeal species encode at least one (and sometimes more than one) NAD^+-dependent DNA ligase. In addition to the haloarchaea, NAD^+-dependent DNA ligases are encoded by the methanogenic euryarchaeal organism *Methanomethylophilus alvus* (Borrel *et al.*, 2012) and potentially also by the newly discovered lokiarchaeota (Spang *et al.*, 2015). Phylogenetic analysis (Poidevin & MacNeill, 2006, Zhao *et al.*, 2006) shows that the genes

encoding these enzymes were most likely acquired as a result of one or more lateral gene transfer events from bacteria at some stage during the evolutionary history of their new hosts.

Using the genetically tractable haloarchaeon *Hfx. volcanii* as a model, we have previously shown that neither the ATP-dependent ligase LigA nor the NAD^+-dependent enzyme LigN is essential for cell growth and division (Zhao *et al.*, 2006). Cells lacking LigA display elevated sensitivity to the DNA damaging agents UV and methylmethane sulfonate (MMS) implying roles for the enzyme in nucleotide excision repair (NER) and base excision repair (BER) pathways, although these have not been further characterised. Cells lacking LigN are no more sensitive to UV and MMS than wild-type, implying a more restricted *in vivo* role for the LigN enzyme compared to LigA. Although neither DNA ligase-encoding gene is essential in *Hfx. volcanii*, simultaneous deletion of both genes is not possible, indicating that the two proteins share an essential function, most likely in the ligation of Okazaki fragments formed during lagging strand DNA synthesis (Zhao *et al.*, 2006).

Here we show that the bacterial NAD^+-dependent DNA ligase inhibitor 2-(cyclopentyloxy)-5'-deoxyadenosine, described in Mills et al. (2011), acts on the LigN protein to inhibit the growth of *Hfx. volcanii* strains that express this DNA ligase alone. LigN inhibition and growth arrest by CPOdA is accompanied by chromosome fragmentation, highlighting the key role the LigN enzyme plays in the maintenance of genome stability in *Hfx. volcanii* cells. CPOdA was originally developed by modifying a compound obtained from high-throughput screening for inhibitors of the NAD^+-dependent DNA ligase encoded by *Haemophilus influenzae* and has been shown to be active against a variety of related enzymes from both Gram negative and Gram positive bacteria, with MICs ranging from 2 µg/ml for *Mycoplasma pneumonia* FH to 64 µg/ml for *E.coli* W3110 (Mills *et al.*, 2011). Interestingly, *Hfx. volcanii* growth inhibition occurs at a significantly lower CPOdA concentration (MIC $\leq$ 50 ng/ml). Whether this is reflected in a corresponding decrease in the IC_{50} for the purified LigN enzyme remains to be seen: other explanations (such as *Hfx. volcanii* having only a limited ability to pump out CPOdA, resulting in a higher intracellular concentration of the compound compared to bacteria) are possible.

To our knowledge, CPOdA has not previously been tested against an archaeal organism that encodes an NAD^+-dependent ligase and indeed, unless the organism lacks – or can be made to lack - an ATP-dependent enzyme, as is the case with the Δ*ligA* strains used here, there appears to be little reason to do so. In addition, CPOdA is presently the only compound that can be used to block the ligation of DNA in the archaea. Because of this, CPOdA has potential utility as a tool for analysis of the role of DNA ligase in archaeal cells, as well as a general inhibitor of S-phase completion. In order to utilise CPOdA as a tool, it is necessary to work exclusively with *Hfx. volcanii* Δ*ligA* strains but as these are straightforward to construct, this should not prove a barrier to further work. In summary, CPOdA represents a new tool in the toolbox for analysis of DNA replication and repair processes in the archaea.

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AUTHOR CONTRIBUTIONS

Stuart MacNeill initiated the study and wrote the manuscript. Xavier Giroux designed and executed the experimental work and edited the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

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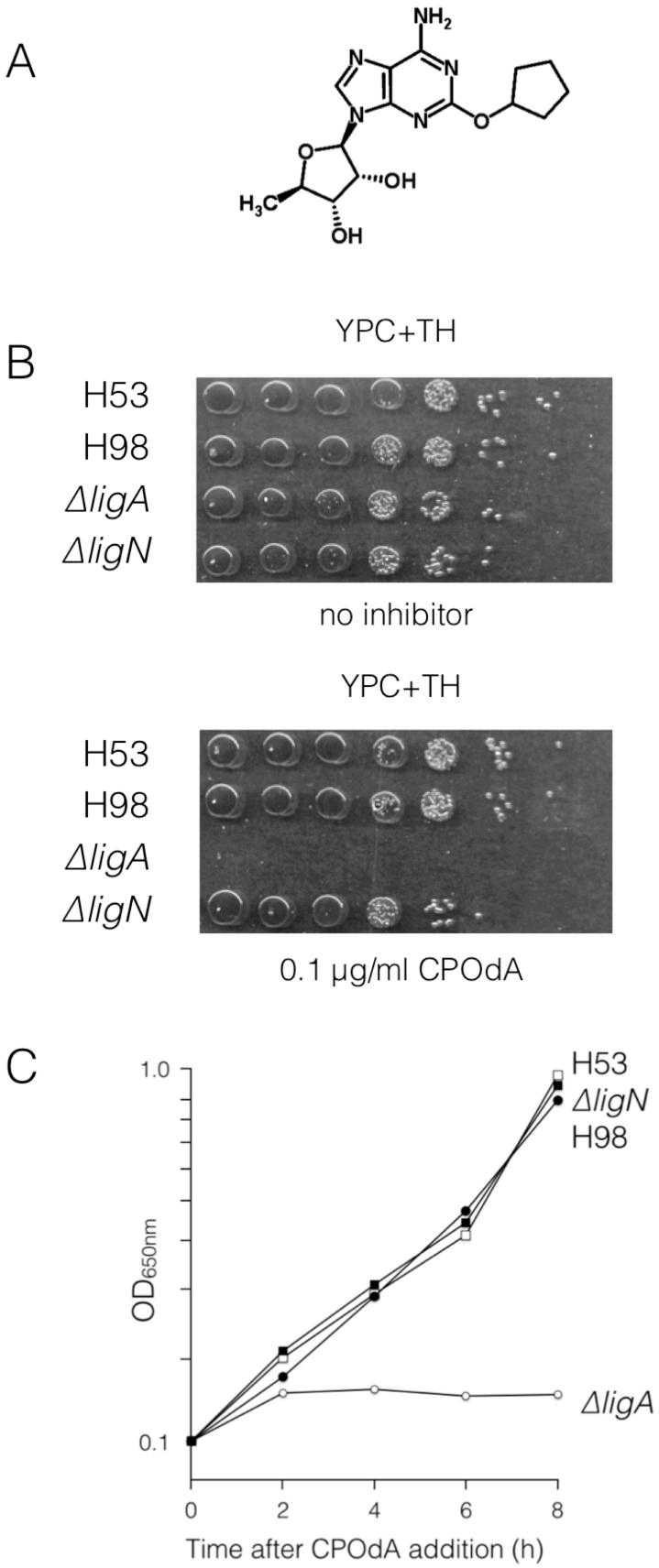
Table 1: *Haloferax volcanii* strains used in this study			
Strain no.	Genotype	Notes	References
SMH628	Δ*trpA* Δ*pyrE2*	H53	(Allers *et al.*, 2004)
SMH892	Δ*hdrB* Δ*pyrE2*	H98	(Allers *et al.*, 2004)
SMH826	Δ*trpA* Δ*hdrB* Δ*pyrE2*	H99	(Allers *et al.*, 2004)
SMH874	Δ*ligA::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2*		(Zhao *et al.*, 2006)
SMH875	Δ*ligN::hdrB* Δ*hdrB* Δ*trpA* Δ*pyrE2*		(Zhao *et al.*, 2006)

Figure legends

Figure 1. A. Structure of 2-(cyclopentyloxy)-5'-deoxyadenosine (CPOdA). **B.** and **C.** Δ*ligA* cells are sensitive to CPOdA on solid and in liquid medium. **B.** Cells were grown in Hv-YPC at 45°C to mid-exponential phase before ten-fold serial dilutions were spotted onto Hv-YPC plates or Hv-YPC plates containing 100 ng/ml CPOdA and grown for 3-5 days at 45°C. **C.** Cells were grown in Hv-YPC at 45°C to mid-exponential phase before CPOdA addition to 0.5 µg/ml at 0 hours. Samples were taken every two hours and growth monitored by measuring optical density (OD) at 650 nm. Key to strains used: Δ*trpA* Δ*pyrE2* (SMH628, H53, open squares), Δ*ligN::hdrB* Δ*trpA* Δ*hdrB* Δ*pyrE2* (filled squares), Δ*hdrB* Δ*pyrE2* (SMH892, H98, filled circles), Δ*ligA::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2* (open circles). Starting OD values were normalised to 0.1 (actual starting ODs in the experiment shown were 0.373, 0.334, 0.500 and 0.251, respectively).

Figure 2. CPOdA addition results in chromosome fragmentation visualised by PFGE. **A.** Exponentially growing wild-type (H98, SMH892, Δ*hdrB* Δ*pyrE2*) and Δ*ligA* (SMH874, Δ*ligA::trpA* Δ*trpA* Δ*hdrB* Δ*pyrE2*) cells treated with 0.5 µg/ml CPOdA for 0, 0.5, 1, 2, 4, 6 and 8 hours. Chromosome plugs were prepared, digested with *Swa*I enzyme and subjected to PFGE as described in the Materials and methods. Fragment sizes are indicated to the right: 1.72 Mb, 1.43 Mb, 230 kb and 82 kb (main chromosome) and 418 kb (pHV3). **B.** Similar analysis of wild-type (H53, SMH628, Δ*trpA* Δ*pyrE2*) and Δ*ligN* (SMH874, Δ*ligN::hdrB* Δ*trpA* Δ*hdrB* Δ*pyrE2*) cells. Note that the larger fragments in strain H53 run as a doublet, indicating that the 1.43 Mb section of the main chromosome in this strain has acquired ~300 kb of additional sequence by unknown means.

Figure 1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OH
H3C
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B
YPC+TH
H53
H98
ΔligA
ΔligN
no inhibitor
YPC+TH
H53
H98
ΔligA
ΔligN
0.1 µg/ml CPOdA
C
1.0
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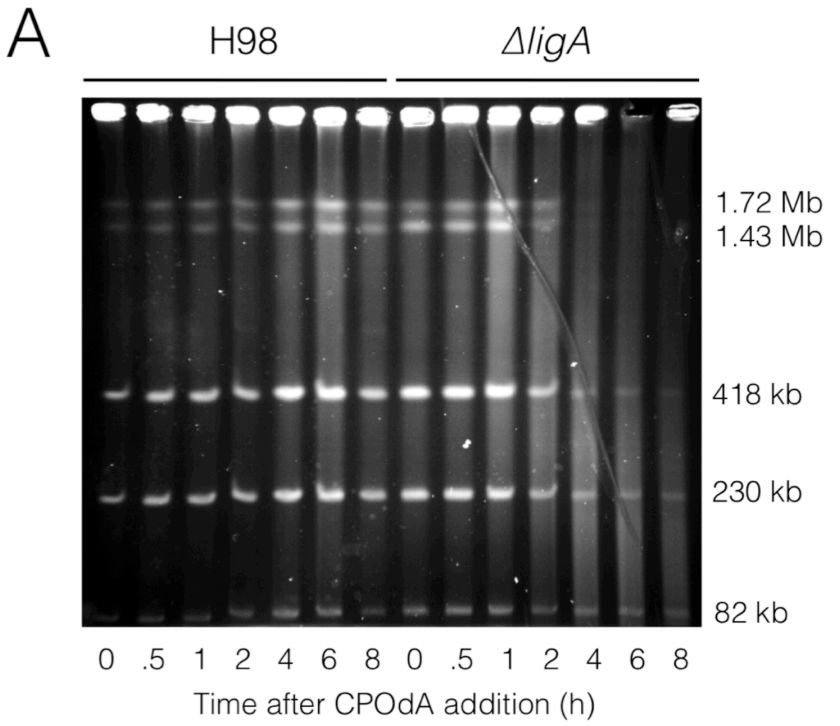
H53
ΔligN
H98
ΔligA
0
2
4
6
8
Time after CPOdA addition (h)

Figure 2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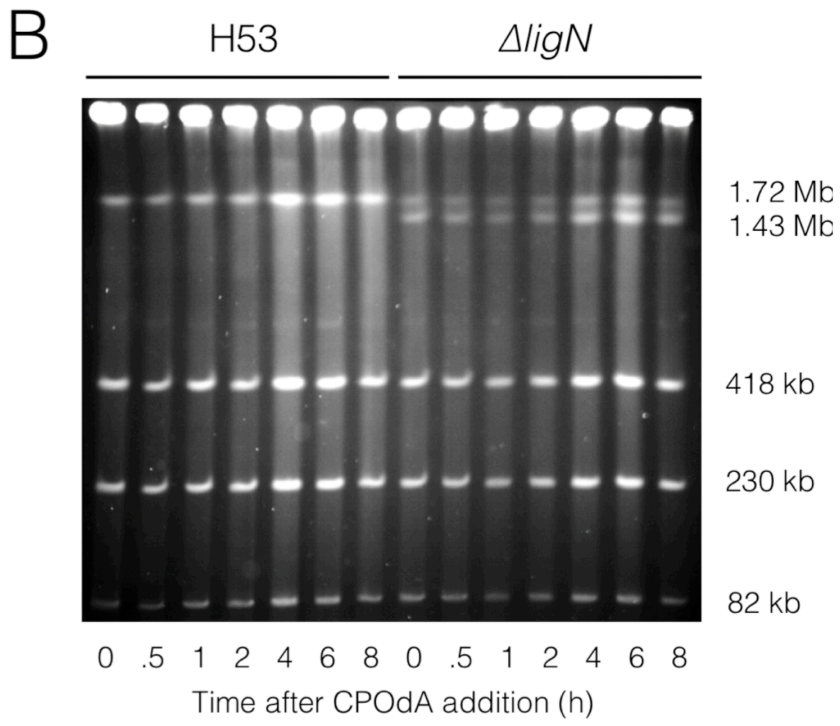
Time after CPOdA addition (h)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H53
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0 .5 1 2 4 6 8 0 .5 1 2 4 6 8
Time after CPOdA addition (h)