RESEARCH ARTICLE

DNA interference by a mesophilic Argonaute protein, CbcAgo
[version 1; peer review: 2 approved with reservations]

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Open Peer Review

Reviewer Status

Invited Reviewers

version 1
published
22 Mar 2019

Report

Report

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Any reports and responses or comments on the article can be found at the end of the article.

Abstract

Background: The search for putative enzymes that can facilitate gene editing has recently focused its attention on Argonaute proteins from prokaryotes (pAgos). Though they are structural homologues of human Argonaute protein, which uses RNA guides to interfere with RNA targets, pAgos use ssDNA guides to identify and, in many cases, cut a complementary DNA target. Thermophilic pAgos from Thermus thermophilus, Pyrococcus furiosus and Methanocaldococcus jasmanii have been identified and thoroughly studied, but their thermoactivity makes them of little use in mesophilic systems such as mammalian cells.

Methods: Here we search for and identify CbcAgo, a prokaryotic Argonaute protein from a mesophilic bacterium, and characterize in vitro its DNA interference activity.

Results: CbcAgo efficiently uses 5’P-ssDNA guides as small as 11-mers to cut ssDNA targets, requires divalent cations (preferentially, Mn^{2+}) and has a maximum activity between 37 and 42 °C, remaining active up to 55 °C. Nicking activity on supercoiled dsDNA was shown. However, no efficient double-strand breaking activity could be demonstrated.

Conclusions: CbcAgo can use gDNA guides as small as 11 nucleotides long to cut complementary ssDNA targets at 37°C, making it a promising starting point for the development of new gene editing tools for mammalian cells.

Keywords
Argonaute, prokaryotic, mesophilic, gene edition, characterization, DNA-DNA interference
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Author roles: García-Quintans N: Investigation, Methodology; Bowden L: Investigation; Berenguer J: Funding Acquisition, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; Mencia M: Conceptualization, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness [BIO2016-77031-R] to J. Berenguer. An institutional grant from Fundación Ramón Areces to the CBMSO is also acknowledged.

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How to cite this article: García-Quintans N, Bowden L, Berenguer J and Mencia M. DNA interference by a mesophilic Argonaute protein, CbcAgo [version 1; peer review: 2 approved with reservations] F1000Research 2019, 8:321 (https://doi.org/10.12688/f1000research.18445.1)

Introduction

Argonaute proteins play a central role in gene silencing and defense against external RNA in eukaryotes, binding small RNA molecules that are then used as guides to scan for complementary RNA targets in the form of mRNAs or RNA viruses. Depending on the presence of specific residues in the protein sequence, the targets can be cut or simply blocked, with degradation carried out by other proteins, leading to inhibition of the expression (silencing) of the genes involved. The structure of eukaryotic Argonaute proteins (eAgo) consists of four domains organized in a specific order (N-PAZ-MID-PIWI) which are each involved in different steps of the protein’s enzyme activity.

Homologues to eAgo that contain these four domains are found in both bacteria and archaea, being collectively known as prokaryotic Argonaute (pAgo) proteins. Their function seems to depend on the species, targeting either RNA or DNA. The best studied pAgos, such as those from *Thermus thermophilus* (ThAgo), *Pyrococcus furiosus* (PfAgo) and *Methanocaldococcus jannaschii* (MjAgo), use ssDNA guides (gDNA) to target DNA in vitro, with ThAgo and PfAgo shown to be involved in defense against invading DNA in vivo. In contrast, pAgo from *Aquific aeolicus* (AeAgo) and *Natronobacterium gregoryi* (NgAgo) use ssDNA guides to target RNA, suggesting a putative role in gene silencing, similar to that of eAgo, or in defense against RNA viruses. Moreover, other pAgos, like that of *Rhodobacter spheroides* (RsAgo), use RNA guides against DNA targets, maintaining its defense capability against invading DNA despite the absence of endonucleolytic activity in its PIWI domain.

High-resolution structures of pAgos in complex with guide and target DNAs support a mechanism of hydrolysis homologous to that of RNase H, in which an Asp-Glu-Asp-Asp catalytic tetrad is formed at the cleavage site of its PIWI domain upon scanning and hybridization of gDNA and target ssDNA. However, the actual mechanism for generation of the gDNA in vivo is essentially unknown and the described in vitro capability of the MjAgo and ThAgo apoproteins to cleave dsDNA (named DNA chopping) seems an unlikely mechanism for the generation of gDNA in vivo, as it cannot explain the observed inactivity against its own genomic DNA.

Following description of the mechanism of action of ThAgo and PfuAgo, the possibility of using pAgos as tools for gene editing has been proposed, with the advantage of being easier to use than the CRISPR-Cas9 system. However, attempts to directly use ThAgo for gene editing of mammalian cells were unsuccessful, likely due to the thermactivity of this protein (unpublished results of our laboratory). Publication of gene editing of mammalian cells using NgAgo, a mesophilic pAgo from a hyperhalophilic archaea, sparked controversy due to the inability of many other laboratories to reproduce the results. Other published research has suggested that the substrates for NgAgo are RNA targets. Despite this, the search has continued for new pAgos that could be successful in gene editing at low temperatures through a DNA-DNA interference mechanism.

Recently, an unreviewed preprint article describing the properties and structure of a mesophilic pAgo derived from *Clostridium butyricum* (CbAgo) has been posted online by the group of John van der Oost. Here we show our independent work leading to the identification of a similar pAgo (CbcAgo) in the strain CWBI 1009 of *C. butyricum*, describing its properties in comparison to that of the CbAgo protein described in the preprint article, including a higher maximum temperature of activity, a strict requirement for 5’-phosphorylated gDNA and a smaller minimum gDNA size required for full activity.

Methods

Identification, overproduction and purification of CbcAgo

The search for mesophilic pAgos was performed using the web interface of the BLASTp program, with the protein sequence of *Natronobacterium georgii* (WP_005580376.1) as a query, and directed to non-redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF, excluding environmental samples from WGS projects. Using the default settings, proteins from two strains of *Clostridium butyricum* were identified (WP_045143632.1 and WP_058142162.1). Further BLASTp and COBALT analysis also with default settings revealed the presence of the four domains that characterize pAgos and the residues required for their likely nuclease activity within the PIWI domain. A fusion gene encoding an N-terminal Strep (II) tag and protein WP_045143632.1 from the strain *C. butyricum* CWBI1009 was synthesized (GenScript) following the codon usage of *E. coli* and cloned into a pET11d vector (Agilent Technologies) to generate a mesophilic pAgo derived from *Clostridium butyricum* (CbAgo) has been posted online by the group of John van der Oost. Here we show our independent work leading to the identification of a similar pAgo (CbcAgo) in the strain CWBI 1009 of *C. butyricum*, describing its properties in comparison to that of the CbAgo protein described in the preprint article, including a higher maximum temperature of activity, a strict requirement for 5’-phosphorylated gDNA and a smaller minimum gDNA size required for full activity.

Proteins purified by this method were separated in an SDS-PAGE gel, digested with trypsin and chemotryptsin and the
resulting peptides were identified by LC-MS/MS in an LTQ Orbitrap Velos Pro (high resolution, short gradient) equipment.

**DNA interference assays**
The synthetic gDNA and ssDNA targets described in Table 1 (SIGMA-ALDRICH) were used for the interference assays (Table 1). The standard interference assays were carried out as follows: after pre-incubation of the CbcAgo protein (6 μM) with a given primer (6 μM), selected among those described in Table 1, for 10 min at 37 °C in reaction buffer (20 mM Tris HCl, 150 mM NaCl, 2 mM of MnCl₂, pH 7.5). The ssDNA target (1.2 μM) was added and the samples were incubated in the same buffer for 50 min at 37 °C, except when otherwise indicated. After incubation, the reaction was stopped by the addition to the sample of one reaction volume of loading buffer (85% formamide, 10 mM EDTA, 20% glycerol, 0.05% bromophenol blue, 0.05% xilencyanol) and further heating for 10 min at 100 °C. Separation of the ssDNA substrate, products and gDNA was carried out by electrophoresis in an 18–20% polyacrylamide gel in the presence of 6M urea (U-PAGE), using SYBR Gold Nucleic Acid Gel Stain for staining (Invitrogen S11494) and a UV spectrophotometer for detection. Synthetic oligonucleotides of different sizes were used as mobility standards.

Assays of nicking activity on dsDNA were carried out following the above protocol using plasmid pMH184 isolated from *E. coli* cells in its supercoiled form as a target (GenJET Plasmid Miniprep Kit, Thermo scientific, Cat no. K0503). The molar ratio between CbcAgo: guide: dsDNA target was 3 : 6 : 0.0074 (μM). Reactions were stopped by adding 100 μg/mL of Pase K (Promega) and the products separated in agarose gels. As mobility standards, linear and nicked forms of pMH184 were generated by digestion with EcoRI (Thermo scientific, FD0275) and *Nt.BspQI* (Biolabs R06445) restriction enzymes, respectively.

**Results**
The Strep II-tagged CbcAgo protein and its inactive DE derivative were overexpressed and purified by affinity chromatography (Figures 1A and 1B). In addition to the wild-type or

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<td>[phos]-CATCCATAACCTCCGCGACCGTTGCAG</td>
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DE mutant proteins, two smaller proteins were co-purified at low proportions even after repeated cycles of affinity chromatography. Mass spectrometry of proteolytic Trypsin/Chemotrypsin digestion fragments of these recalcitrant contaminant proteins revealed them to be the GroEL chaperone and an N-terminal fragment of the Strep II-tagged CbcAgo proteins (Figure 1C). Presence of these co-purified proteins did not interfere with the capacity of the wild-type CbcAgo protein to cleave the ssDNA target after being preloaded with a complementary gDNA guide (Figure 2). As the DE mutant was inactive in these assays, it was concluded that the endonuclease activity detected in the wild type was dependent on the presence of an active endonucleolytic site in CbcAgo’s C-terminal PIWI domain and was not as a result of hidden activity of the co-purified proteins.

Optimization of the DNA-DNA interference activity revealed a strict requirement for divalent cations, with higher activity shown in the presence of Mn\(^{2+}\) compared to Mg\(^{2+}\) (Figure 3), and a...
The wild-type (Wt) and inactive DE mutant (DE) of the CbcAgo protein were pre-incubated with the indicated 5’ phosphorylated guide DNA for 10 min at 37 °C and further used to cut a complementary 45-nucleotide ssDNA target at the same temperature for 1 h. The reactions were carried out in the presence of 2 or 4 mM MnCl$_2$; target (T), guide (G), and the major 34-mer product (P) of the reaction were identified in an 18% U-PAGE gel.

**Figure 2. CbcAgo is active in DNA-DNA interference assays.** The wild-type (Wt) and inactive DE mutant (DE) of the CbcAgo protein were pre-incubated with the indicated 5’ phosphorylated guide DNA for 10 min at 37 °C and further used to cut a complementary 45-nucleotide ssDNA target at the same temperature for 1 h. The reactions were carried out in the presence of 2 or 4 mM MnCl$_2$; target (T), guide (G), and the major 34-mer product (P) of the reaction were identified in an 18% U-PAGE gel.

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<tr>
<td>Guide</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CbcAgo</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
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</table>

**Figure 3. CbcAgo needs divalent cations for activity.** CbcAgo was loaded with a 21-mer, 5’-phosphorylated gDNA (G) and incubated with a complementary 45-mer ssDNA target (T) in reaction buffer in the absence (0) or presence of 2 or 4 mM Mn$^{2+}$ or Mg$^{2+}$. The major 34-mer product (P) of the reactions was identified in an 18% U-PAGE gel; target and guide were the same used in Figure 2.

The requirements of the gDNA were also analyzed, clearly showing a need for a 5’ phosphate end, as gDNAs with 5’-OH were unable to direct the enzyme to the complementary ssDNA target (Figure 6). The cutting site in the ssDNA target was also analyzed in detail using two gDNAs (20- and 21-mers) with a single nucleotide difference at the 5’-phosphorylated end, comparing the size of the largest product of the reaction with ssDNA size markers. As shown in Figure 7, the products obtained had sizes of 33 and 34 nucleotides for the 20- and 21-mer gDNA respectively. The localized cutting site in the target was complementary to the +10 and +11 position with respect to the 5’ end of the gDNA used.

The minimum size of the 5’P-guides was also studied. By shortening the gDNAs at their 3’ end and using them in interference assays against the same ssDNA target, we found similar efficiencies of cleavage up to a gDNA size of 11 nucleotides (Figure 8). Shorter gDNAs of 9- and even 7-mer still allowed the enzyme to partially cut the ssDNA target. Finally, the ability to direct the enzyme activity towards any desired site within a given ssDNA target was also demonstrated, using different guides of the same size but each with hybridization site displaced by a single base with respect to the next. Despite the fact that different efficiencies were detected, the capability of the guides to direct the wild-type CbcAgo activity against any designed site was clearly shown (Figure 9).

Finally, assays on the activity of CbcAgo on dsDNA were carried out using supercoiled forms of plasmid pMH184 using four different guides complementary to the sense and antisense strands of the hygromycin resistance gene. All the gDNAs used were able to direct the production of a nick in the complementary strand, leading to the generation of open circle forms as the main product (Figure 10). However, using a combination of primers complementary to each strand did not result in the generation of linear forms of the plasmid (not shown).

**Discussion**

Novel tools based on CRISPR-Cas9 RNA-DNA interference mechanisms have been developed in the last few years, taking a leading role among the current methods for gene editing. However, significant drawbacks must be overcome in order to allow their safe use in gene therapy, especially off-target actions and putative cellular persistence of the DNA used for the editing process. The description of pAgos in thermophilic bacteria and archaea that are able to target DNA using ssDNA guides has revealed the possibility of developing a new gene editing tool, in which a mix of the protein and its synthetic gDNA would be enough to produce specific cleavage without leaving anything behind that could produce deleterious long-term effects. This possibility was apparently supported by the publication of an article claiming the use of NgAgo to modify several genes in mammalian cell cultures. However, the results were not reproducible by any of several groups. For many microbiologists, the use of a protein from a hyperhalophilic archaea...
Figure 4. Salt tolerance of CbcAgo. Wild-type CbcAgo was preloaded with the same gDNA used in Figure 2 and incubated with the complementary 45-mer ssDNA target in the presence of the indicated concentrations of NaCl (M). Target (T), guide (G), and the major 34-mer product (P) of the reaction were identified in an 18% U-PAGE gel.

Figure 5. Effect of temperature on CbcAgo. Wild-type CbcAgo was preincubated for 10 min at 37 °C with (+) or without (-) gDNA and then used in cleavage assays of an ssDNA target for 1 h at the indicated temperatures. The ssDNA target (T) and gDNA (G) were the same used in Figure 2.

Figure 6. CbcAgo requires 5´-phosphorylated gDNA. The indicated 20- and 21-mer 5´phosphorylated (P) or unphosphorylated (OH) gDNAs were preincubated with CbcAgo and used in interference assays against the same complementary target. Presence (+) or absence (-) of CbcAgo or gDNA in the reaction are indicated. The target (T), guide (G) and the major 34-mer product (P) of the reaction were identified using an 18% U-PAGE gel.
Figure 7. Assessment of the CbcAgo cleavage site. CbcAgo was loaded with 5'-phosphorylated 20- or 21-mer gDNA and used in interference cleavage assays against a 45-mer ssDNA target (T). The sizes of the products (P) were compared with ssDNA standards of the indicated sizes, using a 20% U-PAGE gel, leading to the conclusion that the cleavage site was complementary to the 10-11 base position of the gDNA.

Figure 8. Minimum size of gDNA used by CbcAgo. (A) CbcAgo was incubated with 7-, 9-, 11-, 13-, 15-, 17- or 19-mer gDNAs complementary to a ssDNA target and used them in interference cleavage assays. The target (T), guide (G) and major product (P) of the reaction were identified using an 18% U-PAGE gel. (B) Sequences of the gDNAs and target ssDNA used in (A).
Figure 9. Selection of the cleavage site by CbcAgo. CbcAgo was pre-loaded with a collection of 21-mer, 5′-phosphorylated gDNA that paired at positions displaced by a single nucleotide with a 45-mer ssDNA target (T). The products of the reactions were compared using a 20% U-PAGE gel with ssDNA markers of the indicated sizes (mer). (A) Assays with w1 to w4 gDNA. (B) Assays with gDNA w-5 to w-8. (C) Target DNA and gDNA used in A and B. Note that the cutting site was displaced along the target by a single nucleotide, always pairing at position 10-11 of the gDNAs (shaded triangle).

was suspect as they have evolved at the sequence level to tolerate very high potassium chloride concentrations as intracellular compatible solutes. Despite this, the article sparked a growing interest in finding an appropriate pAgo protein that could have this function, as thermophilic pAgos have little or no activity at mesophilic temperatures. In this context, the search for a mesophilic pAgo led our group (and that of J. van der Oost) to independently focus on a pAgo from Clostridium butyricum due to the mesophilic character of this organism and the presence of a pAgo in its genome that contains the catalytic tetrad at its PIWI domain. In a preprint article posted online, the group of J. van der Oost provided an...
exhaustive description of the CbAgo protein but did not iden-
tify the exact strain they used. Although most of the findings of
that article coincide with the properties of the CbcAgo protein
described here, there are also some relevant differences that
could relate either to differences in the sequence of the proteins
or to the different methods used for the characterization.

Firstly, in our study the CbcAgo protein seems slightly more sta-
ble, as we detected significant cleavage capacity of a ssDNA tar-
get at 55°C, whereas the preprint article described a 50°C limit
for the activity of CbAgo. This could be related to differences
in the protein sequence, as commented above, or to the presence
of a chaperone (GroEL) in our samples that possibly protects
the protein at 55°C. However, the main differences we found
were concerning the requirement for 5´ phosphorylation of the
gDNA (Figure 4) and the minimum gDNA size required for
efficient cleavage (Figure 5). In our study, the requirement for
phosphorylation of the gDNA was quite strict and no posi-
tive cutting was detected with any of the 5'-OH gDNAs used in
different assays. This result is in agreement with the reported
requirements of other pAgos such as ThAgo and PfAgo for gDNA phosphorylation.
Also, the minimum size for gDNA to be as efficient as longer gDNA in directing enzyme activity
was 11 nucleotides, although we also detected some activity for
gDNAs as short as 7 nucleotides (Figure 8). This contrasts with

Figure 10. Effects of CbcAgo on dsDNA. (A) CbcAgo was pre-loaded for 15 min at 37°C with the Hygro-1 to 4 guides (lanes 1 to 4) and
incubated for 16 hours at the same temperature with plasmid pMH184 that was essentially supercoiled (lane 5). Linear plasmid and nicked
open circle were run in lanes 6 and 7 respectively. The molar ratio between CbcAgo:guide:target was 3 μM: 6 μM: 0.0074 μM. Reactions were
stopped by adding 100 μg/mL of Proteinase K (Promega) and the products separated in agarose gels. (B) Sequence of the target in plasmid
pMH184 paired with the gDNAs used in panel A.

Putting our data in the context of the structure for CbcAgo
described in the figures of the preprint by the group of J. van der
Oost, and in that of ThAgo once even small gDNA are
attached at a position of the MID domain defined by the gDNA
5P-residue, CbcAgo is able to scan ssDNA for a matching
sequence, approximating paired positions 10–11 to the active
site of the PIWI domain, where the ssDNA target is cleaved in
a cation-dependent manner. Smaller guides (i.e. 7-mers) may
also function in the scanning, being only partially successful in
guiding the substrate to the active site of the enzyme. This
result supports the idea that CbcAgo needs a preloaded
guide to fix its position on the target ssDNA and cut it
efficiently, although the effectiveness of this cleavage seems to
be related to the structure of the primer at the designated reaction
temperature.
Another discrepancy has to do with the effects of CbcAgo on dsDNA. In this study, only nicking activity was detected when supercoiled plasmids were used as substrates (Figure 10). In fact, we did not detect the linearized plasmid form, even when two gDNAs (one for each strand) were used. However, in the preprint article\(^4\), plasmid linearization was detected at low yields, the activity being much more efficient in regions with low G+C content. This suggests that the CbcAgo protein alone is unable to open dsDNA after its nicking activity and that future directed evolution work will be needed for better adaptation to this type of substrate.

**Data availability**

**Underlying data**

Open Science Framework: *Clostridium butyricum* CWBI1009 Ago. [https://doi.org/10.17605/OSF.IO/8GQUZ]\(^6\)

This project contains raw images of the gels used for each figure.

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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**Grant information**

This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness [BIO2016-77031-R] to J. Berenguer. An institutional grant from Fundación Ramón Areces to the CBMSO is also acknowledged.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Acknowledgements**

Technical assistance of E. Sánchez is acknowledged. The professional editing service NB Revisions was used for technical preparation of the text prior to submission.
Open Peer Review

Current Peer Review Status:  ?  ?

Version 1

Reviewer Report 02 August 2019

https://doi.org/10.5256/f1000research.20180.r51940

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Nathan A. Tanner  
New England Biolabs, Ipswich, Massachusetts, USA

The study from Garcia-Quintans et al. looks at a new pAgo protein from Clostridium and investigates factors influencing its cleavage activity on DNA substrates. The in vitro activity is characterized pretty well, but there are some serious issues I find with the data and its presentation, as well as the contradictory findings of another recent publication.

1. The authors report a significant (by eye ~40%) contaminant of an N-terminal truncation at about half the size of the expected protein (Figure 1). I would assume this is an inactive form of the enzyme, but does it still bind guides? Bind to DNA targets? Perhaps affect the results of all the experiments in the paper? This should be addressed in more detail, and ideally cleaned up (along with the GroEL contaminant) using another chromatography step.

2. Most of the gels are shown as zoomed in cropped sections of the gel. I feel these should instead show the whole, or at least more of, the gel, and include low-molecular weight marker standards. Some gels have oligonucleotide standards but the resolution is very poor in terms of distinguishing between a few bases (I’d suggest moving the guides by more than 1 base). And as shown in Figure 8 11 ntd ssDNA can clearly be seen, but where is it in the other gels where the product should be 2 ssDNA’s? The most problematic is Figure 5 where the far right gel is too poor for publication, and seems to show production of P species without added guide at 55C? Where is the guide in all those wells? Figure 8 seems to have additional bands between P and guide, Figure 10 has an unidentified high molecular weight species, and the size markers in Figure 7 should be labeled more clearly.

3. I feel there should be more explanation given to the (to me) bizarre finding that a 7 or 9 base guide can cut at the +10/11 position...which of course does not have a guided complement. How do the authors think this can happen?

4. The authors mention the Hegge at al., preprint, which they should, but that paper was published in NAR after this study. And importantly, so was another study with CbAgo, from a strain mentioned here (Kuzmenko et al). In this study, the authors show several things at odds with the current work: no cleavage with 10 or 12-base guides even after 24hr incubation, activity to 60C, ability to
use 5'-OH guides, the ability to cut dsDNA with opposite strand guides at 37°C in 1-4h, and with moderate (500 nM) concentrations of CbAgo a chopping activity on plasmid DNA. It is likely this work was not available at the time the reviewed study was published, but it is difficult to ignore the contradictions now. It is possible that the Cb/CbcAgo protein is exactly the same in all 3 studies, and these discrepancies are significant for the conclusions presented here.

5. Related, I'd expect there to be some plasmid chopping given the time and concentrations the authors describe. But no Apo reactions are shown in Figure 10, an important control that is left out. And a comparison of attempts to digest non-supercoiled plasmid would be good for the explanation that dsDNA cannot be accessed w/o supercoiling.

6. Minor points, but there are some errors ("xilencyanol", "ImajeJ) and inconsistencies (PifAgo/PfuAgo) that should be fixed.

References

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** DNA enzymes and polymerases, biochemistry and molecular biology, biotechnology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
García-Quintans et al. reports on the identification of an Argonaute protein coding gene from the mesophilic bacterium Clostridium butyricum strain CWBI 1009, and characterise its product as a potential genome editing tool alternative to the well known CRISPR/Cas. Authors have purified the native protein and an inactive variant as a control, and thoroughly characterise its activity in relation to a number of parameters such as different temperatures, cations and ionic strength. Altogether, the work of García-Quintans is a well-designed characterisation of the in vitro activity of the CbcAgo protein.

A similar work has been recently published in Nucleic Acids Research, by the group of John van der Oost using a similar CbAgo protein from an unspecified strain of C. butyricum, as acknowledged by the authors. It appears that both proteins have very similar characteristics.

The authors claim some relevant differences between both proteins:

**Enzyme stability at different temperatures:** In first place, none of authors assay thermostability, they assay activity at different temperatures, which is not the same. This should be changed in the Discussion, pg. 10. Based on the partial activity detected at 55°C in this paper, authors claim CbcAgo might be more “stable” than CbAgo (Discussion, pg. 10). However, CbAgo activity was not assayed at 55°C but at 50°C (partially active) and 64°C (inactive). A comparison of the activities at the same temperature, 50°C, which show almost maximal activity of CbcAgo but substantially reduced activity of CbAgo (<50%), would be more reliable. Anyways, since the difference is very small, it is very difficult to ascertain whether the differences are real or a consequence of slightly different assay conditions.

**Strict dependence on phosphorylation:** Both CbAgo and CbcAgo, were unable to cut a short 45-mer target DNA if the gDNA is 5’-OH. However, Hegge et al., 2019, additionally reported partial activity of CbAgo on a longer target (120-mer), which was not tested in this manuscript. Therefore, in this regard, there is no data that supports the difference between both proteins claimed in this manuscript.

**Minimum size length of the gDNA:** By comparing the results obtained with both proteins, it is apparent that CbcArgo requires a shorter gDNA to cleave the target. I think this is the most evident difference. However, as the authors acknowledge, these differences may be due to technical reasons rather than a difference in catalytic activity between the Argo proteins. The question of whether CbcArgo and CbArgo show any difference in activity could only be solved by making a side by side comparison of both proteins having the same tag, and using exactly the same procedure.

Anyways, I really miss an alignment of CbArgo and CbcArgo proteins to know how different these proteins are at the amino acid level.
Other comments:
Please, properly align lanes and lanes names/numbers in Fig. 1.
Requirement for 5´ phosphorylated gDNA is shown in Fig. 6, not 4 (Discussion, pg. 10).

References

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Bacterial Molecular Biology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.
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