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ORIGINAL PAPER

Genetic analysis of the Holliday junction resolvases Hje and Hjc in *Sulfolobus islandicus*

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Abstract The in vivo functions of Hje and Hjc, two Holliday junction resolvases in *Sulfolobus islandicus* were investigated. We found that deletion of either *hje* or *hjc* had no effect on normal cell growth, while deletion of both *hje* and *hjc* is lethal. Although Hjc is the conserved resolvase in all archaea, the *hje* deletion rather than *hjc* deletion rendered cells more sensitive to DNA-damaging agents such as hydroxyurea, cisplatin, and methyl methanesulfonate than the wild type (WT). Intriguingly, the sensitivity of Δ*hje* could not be rescued by ectopic expression of Hje from a plasmid and Hje overexpression slowed growth and large cells appeared with more than two genome equivalents. We showed that Hje was maintained at a low level in WT cells. Furthermore, transcriptomic microarray analysis revealed that the abundance of transcripts of many genes including those involved in DNA replication, repair, transcription regulation, and cell division changed drastically in the Hje-overexpressed strain. However, only limited genes were up- or downregulated in the *hje* deletion strain. Our findings collectively suggest that Hje is the primary resolvase involved in DNA repair and its expression must be tightly controlled in cells.

Keywords Archaea · Holliday junction resolvase · Hje · Hjc · DNA repair

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Introduction

Double-stranded DNA break (DSB) is one of the most deleterious damages to cellular DNA. DSBs can be repaired through homologous recombination (HR) which generates a four-way branched DNA intermediate referred to as Holliday Junction (HJ) (Heyer et al. 2003; West 2009). HJ can also be generated by replication fork regression (Manosas et al. 2012). For cells to survive, covalently linked harmful intermediates must be processed by enzymes including helicase (replication fork reversal), helicases/topoisomerase complexes, or structure-specific endonucleases including HJ resolvases (Schwartz and Heyer 2011).

Canonical HJ-resolving enzymes, HJ resolvases, catalyze HJ resolution by introducing symmetrical nicks in two strands, producing nicked duplex products that can be ligated without further processing. HJ resolvases have been identified and characterized in bacteria, archaea, and Eukarya and have been classified into several groups based on sequence conservation (Aravind et al. 2000). *E. coli* RuvC, the best-characterized HJ resolvase, belongs to the integrase family, functioning with RuvA and RuvB for processing recombination intermediates (Dunderdale et al. 1991; West 1997). RecU from Gram-positive bacterium *Bacillus subtilis* belongs to the nuclease family (McGregor et al. 2005). Eukaryotic resolvases GEN1/Yen1 family have only recently been identified and characterized and were found to be members of the Rad2/XPG endonuclease

family (Ip et al. 2008; Svendsen and Harper 2010). GEN1/Yen1 share striking similarity with respect to amino acid sequence and HJ cleavage with archaeal and Gram-positive bacterial resolvases and they promote HJ resolution similar to *E. coli* RuvC (Ip et al. 2008). More recent studies indicate that GEN1 and Yen1 are regulated by different mechanisms (Chan and West 2014; Matos and West 2014).

All archaea have an HJ resolvase Hjc (Holliday junction cleavage) (Komori et al. 1999). Some archaea including most *Sulfolobus* and their relatives *Acidianus* and *Metallosphaera* harbor a second HJ resolvase named Hje (Holliday junction endonucleases) which is known to be related to Hjc (Kvaratskhelia and White 2000a, b). Biochemical and structural properties of Hjc and Hje have been well documented (Komori et al. 1999; Kvaratskhelia and White 2000a, b; Bond et al. 2001). Hjc and Hje differ in the dimer orientation which may cause different cleavage patterns against a fixed HJ (Kvaratskhelia and White 2000a, b; Nishino et al. 2006). Hjc and Hje share the same fold to the type II endonucleases. However, limited reports describe the genetic analysis of archaeal resolvases (Fujikane et al. 2010; Lestini et al. 2010), so the in vivo functional relationship between Hjc and Hje is obscure.

Here, we used a genetic system developed in *S. islandicus* (Deng et al. 2009; Zhang et al. 2010) to investigate in vivo functions of *hje* and *hjc*. Mutants of *hje* and *hjc* were generated and strain sensitivity to DNA-damaging agents was evaluated. In the presence of hydroxyurea (HU), methyl methanesulfonate (MMS), and cisplatin, Δ*hje*, but not Δ*hjc,* was more sensitive. Strains overexpressing Hje had growth defects and changes in gene transcripts including those involved in DNA replication, repair, transcription regulation, and cell division, suggesting that Hje expression must be strictly controlled in *Sulfolobus* cells.

Materials and methods

Strains and growth conditions

Host strain *S. islandicus* Rey15A(E233S)(Δ*pyrEF*, Δ*lacS*) ('SisE233S' hereafter) was grown at 75 °C in rich medium (MTSyV) containing mineral salts (M), 0.2 % (wt/vol) tryptone (T), 0.2 % (wt/vol) sucrose (S), 0.05 % (wt/vol) yeast extract (Y), and a mixed vitamin solution (V) supplemented with 0.01 % (wt/vol) uracil (U), as described previously (Zhang et al. 2010). The medium without yeast extract and uracil (MTSV) was used for screening and purification of the transformants. MTSyV supplemented with 5′-fluoroorotic acid (5-FOA, 50 μg/ml) was used for counter selection of deletion mutants. For sensitivity assays, the indicated amounts of DNA-damaging agents were added to the rich medium. The medium was solidified with 1.2 % (wt/vol) Phytagel (Sigma, St. Louis, MO, USA) as required.

Construction of deletion and overexpression plasmids

The deletion plasmids for *hje* (SiRe_0930) and *hjc* (SiRe_1431) were constructed based on the genomic sequence of *S. islandicus* Rey15A and according to previously described methods (Zhang et al. 2010; Guo et al. 2011). The fragments containing *hje* and *hjc* and the corresponding flanking sequences were amplified by PCR, digested by restriction enzymes, and inserted into pMID to obtain the plasmid used for gene knockout. The primers used for PCR are listed in Supplementary Table 1.

To construct overexpression vectors pSeSD-Hje-C-His and pSeSD-Hje, *hje* was amplified by PCR and digested with *Nde*I/*Sal*I, and the resulting fragments were ligated to pSeSD (Peng et al. 2012) digested with the same enzymes. By PCR, the original stop codons in the genes were removed or maintained, allowing expression of Hje protein with a six-histidine tag at the C-terminal or without a tag, respectively. To construct the expression vector pSeSD-NPHje-C-His and pSeSD-NPHje, a fragment (5′ upstream region, 305 bp) containing *hje* and its native promoter was amplified by PCR. After digestion with *Sph*I/*Sal*I, the resulting fragment was inserted into pSeSD digested with the same restriction enzymes. The plasmids used and constructed in this study are listed in Table 1.

Construction of strains

S. islandicus strain SisE233S or the knockout strain was transformed with the overexpression plasmid or

Table 1 Plasmids used in this study

Plasmids	Features	Source or reference
pMID-*hje*	pUC19 carrying L-, R- and G-arm of *hje* gene plus the marker cassette (*pyrEF*+*lacS*)	This work
pMID-*hjc*	pUC19 carrying L-, R- and G-arm of *hjc* gene plus the marker cassette	This work
pSeSD	*E. coli/Sulfolobus* shuttle vector harboring the marker *pyrEF*	Peng et al. (2012)
pSeSD-Hje, pSeSD-Hje-C-His	pSeSD carrying *hje* genes with *araS* promoter coding for Hje or Hje-C-His	This work
pSeSD-NPHje, pSeSD-NPHje-C-His	pSeSD carrying *hje* genes with native promoter coding for Hje or Hje-C-His	This work

Table 2 Strains used in this study

Strains	Genotype	Source or reference
S. islandicus REY15A (SisE233S)	Δ*pyrEF*Δ*lacS*	Deng et al. (2009)
Δ*hje*	*hje* deletion mutant	This work
Δ*hjc*	*hjc* deletion mutant	This work
SisE233S-Hje-C-His	SisE233S with the genome-coded wild type Hje replaced with a His-tagged Hje	This work
Δ*hje*/pSeSD-Hje, Δ*hje*/pSeSD-NPHje	Δ*hje* with *hje* on the expression vector pSeSD harboring *araS* promoter or native promoter	This work
Sis/pSeSD-NPHje-C-His	SisE233S with *hje-C-His* on the expression vector pSeSD harboring native promoter	This work
Sis/pSeSD-SisEstA-C-His	SisE233S with *SisEstA-C-His* on the vector pSeSD harboring *araS* promoter	Mei et al. (2012)
Sis/pSeSD-Hje, Sis/pSeSD-Hje-C-His	SisE233S with *hje* or *hje-C-His* on the vector pSeSD harboring *araS* promoter	This work

linearized knockout plasmid (pMID) DNAs by electroporation, according to the method described (Deng et al. 2009). Transformed strains were selected and purified by several rounds of screening on solid MTSV medium. The purity of the merodiploid strain was confirmed by X-gal staining and PCR analysis. The deletion strain was obtained by several rounds of counter selection with MTSyV medium containing 5′-fluoroorotic acid (5-FOA) and uracil, and the purity of the deletion strains was confirmed by PCR using the flanking primers. *Hje/hjc* double deletion was performed by transformation of Δ*hjc* with pMID-*hje* and subsequently the selection and counter selection. The overexpression strains were confirmed by plasmid extraction and digestion with restriction enzymes and sequencing. A marker cassette containing *hje-C-his* and *pyrEF-lacS* was transformed into Δ*hje*, generating the strain SisE233S-Hje-C-His which encoded C-terminal His-tagged Hje by the chromosomal gene. The strains used and constructed in this study are listed in Table 2.

Sensitivity assays

Strains with the same cell vitality were obtained by several rounds of inoculation and cultivation. When the cultures reached optical density at 600 nm (OD_{600}) of 0.4, tenfold serial dilutions (in rich medium) were spotted onto solid-rich medium supplemented with various concentrations of MMS, HU, and cisplatin. The plates were photographed after 5–6 or 12–14 day incubations. For survival assays on 2 mM HU plates, 100 µl of the 10^{-3} and 10^{-4} dilutions of SisE233S and 10^{-3} and 10^{-2} dilutions of Δ*hje* cells were plated, whereas for survival assays with 0.5 mM and 1 mM HU, 10^{-5} and 10^{-4} dilutions of both control and Δ*hje* were plated. Colonies were counted after 5–14 days of growth at 75 °C. All DNA-damaging chemicals were purchased from Sigma (St. Louis, MO, USA).

Growth curves and doubling time measurements

To obtain growth curves, cells were grown to early log-phase and then the OD_{600} was monitored every 6 or 12 h. To measure generation time, cells were cultured to OD_{600} of 0.2 and diluted 8- or fourfold in rich medium. The time required for diluted cells to grow to OD_{600} ~ 0.2 was used to calculate the doubling time. Values were obtained from three independent experiments.

Western blot

SisE233S-Hje-C-His or those containing various Hje-C-His overexpression plasmids were cultured in one L MATV (arabinose instead of sucrose) or MSTV medium until the cell density (OD_{600}) reached ~0.8. Cells were harvested and disrupted by sonication in 15 ml of buffer (50 mM Tris–HCl, pH 8.0, and 100 mM NaCl). Cell debris was removed by centrifugation (10,000 rpm for 30 min at 4 °C) and the cell extract or the purified proteins after Ni^{2+} affinity chromatography were separated by 15 % SDS-PAGE. Gel proteins were transferred to polyvinylidene difluoride membranes which were incubated with anti-6 × His antibodies under standard Western blot conditions. Bands were detected with HRP-labeled goat anti-mouse IgG and an image analyzer (ImageQuant 400, GE Healthcare, Buckinghamshire, UK).

Flow cytometry

Flow cytometry was performed on an Apogee A40 flow cytometer. Cells were grown to early log-phase (OD_{600} of 0.2–0.3) and fixed in 70 % (vol/vol) ethanol at 4 °C for at least 12 h. Before DNA staining, cells were centrifuged at 2,800 rpm for 20 min at 4 °C. The supernatants were removed, and the resulting pellets were resuspended in 1 ml Tris–$MgCl_2$ (10 mM Tris, 10 mM $MgCl_2$, pH 7.5). Cells were centrifuged again at 4 °C

and resuspended in 150 µl of the same buffer containing fresh staining buffer (20 µg/ml EB and 100 µg/ml mithramycin A). Stained cells were kept on ice for about 60 min before analysis.

Microarray

SisE233S strains harboring pSeSD or pSeSD-Hje-C-His were cultured in 50 ml of arabinose medium and collected when OD_{600} reached ~0.2. Total RNA was isolated according to the standard TRIZOL protocol and RNA quality was determined by measuring the UV absorbance at 260 nm. cDNA was prepared with 25–30 µg of RNA and anchored oligo (dT), and labeled with Cy-dyes according to the protocol of Amersham CyScribe post-labeling Kit (GE) with slight modification. cDNA from each strain was labeled with both Cy3 and Cy5. Dye-swap was performed to correct error caused by different Cy-dyes (Li et al. 2011). The microarray was designed to target open reading frames (ORFs) of the genome of *S. islandicus* Rey15A and various endogenous and exogenous genetic elements of *Sulfolobus*. Each microarray consisted of 2,609 oligonucleotides (50 nucleotides in length) that were spotted onto glass slides. Microarray hybridization was performed, as previously described (Li et al. 2011). Microarray slides were scanned by GenePix 4100A Microarray scanner (Molecular Devices). Microarray analysis of the *hje* deletion strain was performed as described above except that SisE233S was used as the reference.

Results

Deletion of either *hje* or *hjc* does not affect cell growth, but *hje/hjc* double deletion is lethal

To understand in vivo functions of Hje and Hjc, single deletion mutants were constructed in crenarchaeon *S. islandicus* Rey15A (E233S) (Δ*pyrEF*, Δ*lacS*) using recently developed genetic tools, which allow markerless gene knockout and inducible expression of proteins in the presence of sugar-inducer sucrose versus arabinose (Zhang et al. 2010; Peng et al. 2012). Gene deletion was confirmed by PCR (Fig. 1a). To determine whether *hje* or *hjc* deletion changed cell growth, we measured mutant and WT (SisE233S) strain doubling time. As shown in Fig. 1b, deletion and WT strains were not different with respect to doubling time and growth curves of SisE233S and mutants did not differ (data not shown). Thus, deletion of *hje* or *hjc* did not affect growth under normal culture conditions.

Since there are two HJ resolvases, Hje and Hjc, we attempted to construct a *hje/hjc* double deletion mutant to test whether they have redundant functions in HR. Using the two-step gene knockout system, we obtained an integrated strain with the *pyrEF-lacS* marker cassette and homologous arms inserted into the *hjc* locus in *hje* deletion mutant (Supplementary Fig. S1a, b). After counter selection on plate containing 5-FOA and uracil, most colonies turned blue after X-gal staining (Supplementary Fig. S1c).

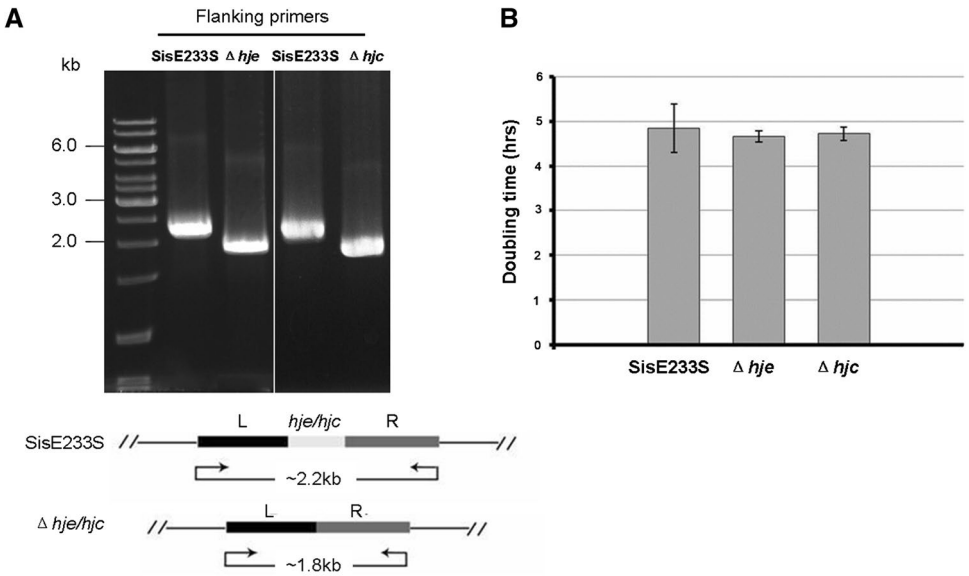

Fig. 1 Deletion of either *hje* or *hjc* did not change cell growth. **a** Confirmation of gene knockout in Δ*hje* and Δ*hjc* strains by PCR. Genomic DNA was isolated from cells and used for PCR with the flanking primers. No band corresponding to the wild type (SisE233S) could be amplified. Using gene-specific primers, no band corresponding to SisE233S could be amplified (not shown). **b** Strains of SisE233S, Δ*hje*, and Δ*hjc* had similar doubling times. Strains were grown in rich medium (MTSyVU) and doubling time was measured (see "Materials and methods"). Means and standard deviations were calculated based on three independent experiments

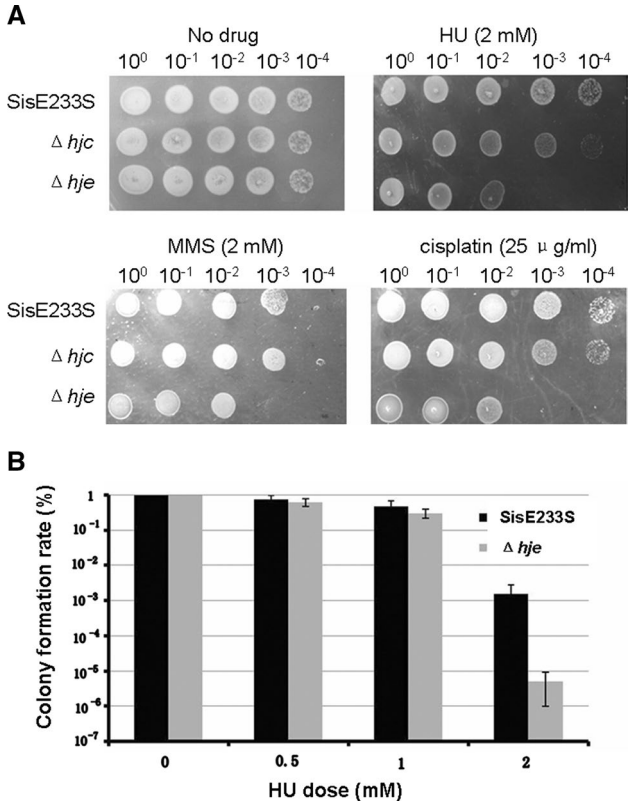

Fig. 2 The Δ*hje* strain was more sensitive to DNA-damaging agents HU, MMS, and cisplatin than SisE233S. **a** Cells of SisE233S, Δ*hje*, and Δ*hjc* strains were grown to OD_{600} ~ 0.4, and tenfold serial dilutions were spotted on solid medium supplemented with the indicated DNA-damaging agents and cultured for 8–10 days at 75 °C. **b** Colony formation rate of SisE233S and Δ*hje* cells in the presence of various concentrations of HU. Colonies were counted after 6–11 days' incubation on solid medium. Means and standard errors from three independent experiments are shown

PCR analysis on the genotypes of a few white colonies confirmed that *hjc* together with the marker had not been deleted and point mutation had occurred in *pyrEF* gene (Supplementary Fig. S1d). Furthermore, we also failed to get any colony deleted for *hje* in Δ*hjc* background (data not shown). Our results revealed that a *hje/hjc* double deletion is lethal in *S. islandicus*.

Deletion of *hje*, not *hjc,* increased cell sensitivity to DNA-damaging agents

To investigate roles of *hje* and *hjc* in DNA repair, we measured sensitivity of Δ*hjc* and Δ*hje* to DNA-damaging agents HU, cisplatin, and MMS, which can cause replication fork-stalled and DNA double-strand breaks. The *hjc* deletion strain was not more sensitive to MMS (2 mM) and cisplatin (25 μg/ml) than SisE233S, although it was slightly more sensitive to HU (2 mM) (Fig. 2a). In contrast, the *hje* deletion strain was more sensitive to the tested DNA-damaging agents than SisE233S cells.

To understand the effect of DNA-damaging agents on Δ*hje*, we measured colony formation ability of Δ*hje* and SisE233S in the presence of various concentrations of HU (0, 0.5, 1.0, and 2 mM). As shown in Fig. 2b, at low concentrations (0.5 and 1 mM), HU slightly affected colony formation of Δ*hje* and SisE233S on the solid medium, but this was not significantly different. When HU was increased to 2 mM, cell viability of both strains was drastically reduced, and the effect on the mutant was more pronounced. Thus, Hje plays a more direct role in DNA repair than Hjc.

Complementation of Hje deficiency by ectopic overexpression from a plasmid resulted in a negative effect on cell growth

Because Δ*hje* growth was obviously slowed after HU treatment, we analyzed whether ectopic expression of Hje could rescue genotoxin sensitivity. To do this, we constructed plasmid pSeSD-NPHje carrying *hje* with its native promoter and pSeSD-Hje carrying *hje* with an *araS* promoter, and transformed them into Δ*hje*. Strains harboring an empty vector pSeSD, recombinant vectors pSeSD-NPHje and pSeSD-Hje were assayed for sensitivity to HU. Surprisingly, the strain carrying either pSeSD-NPHje or pSeSD-Hje did not show any resistance to HU (Fig. 3). On the contrary, both complementing strains were more sensitive to HU than the control. Because the copy number of pSeSD in the cells is 3–5 in the late exponential-growth phase (Deng et al. 2009, Peng et al.

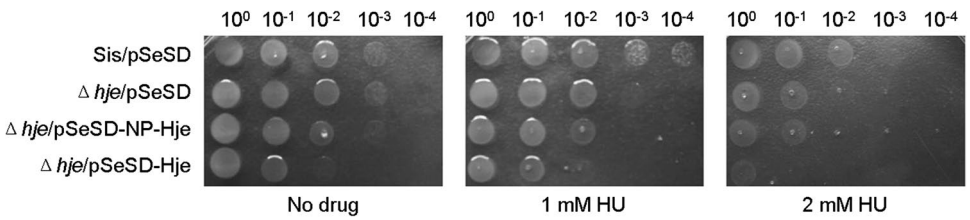

Fig. 3 Complementation of Δ*hje* deficiency in sensitivity to DNA-damaging agent HU by ectopic expression of Hje. In strain Δ*hje*/pSeSD-Hje and Δ*hje*/pSeSD-NPHje, Hje was expressed under the control of the arabinose inducible promoter or the native promoter (NP) of Hje. The plate was made with MTAV medium

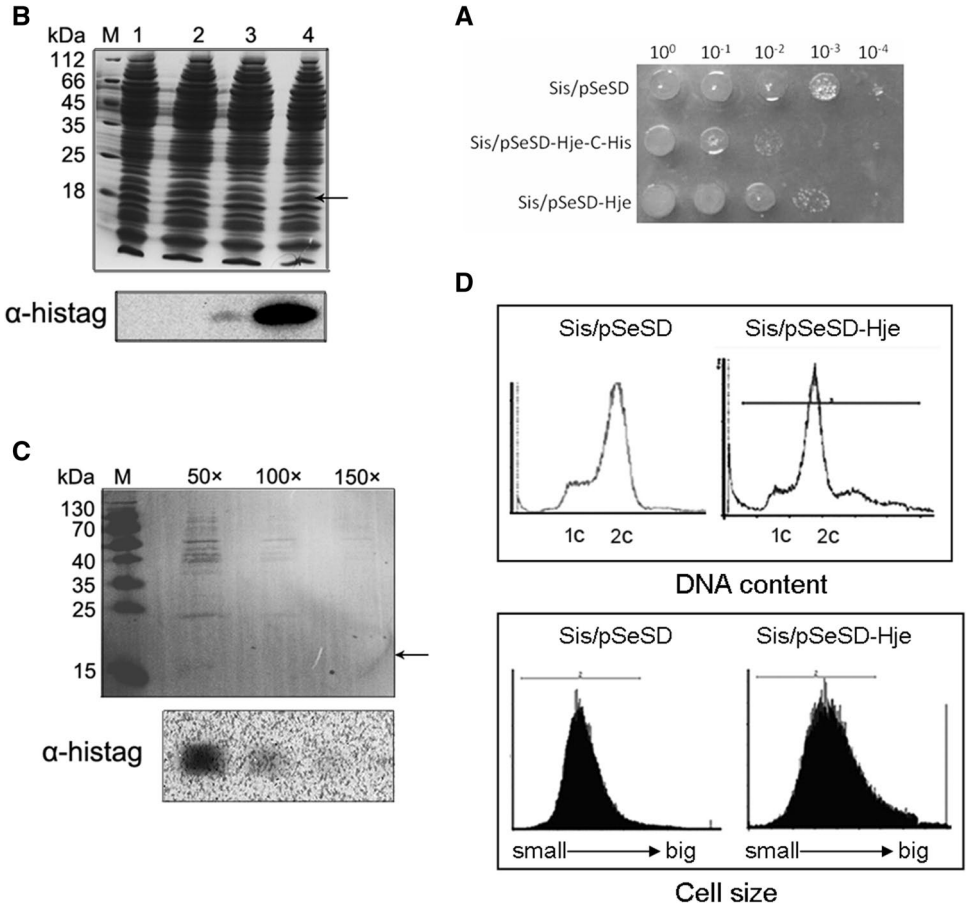

Fig. 4 Hje was low and Hje overexpression caused growth defects in wild-type cells. **a** Overexpression of Hje in E233S caused growth defects. The plate was made with MTAV medium in which arabinose was used as the carbon source to induce protein expression. Plates were photographed after incubation for 14 days. **b** Assay of Hje expression in different strains. Whole-cell extracts from 20 μl cultures at OD_{600} ~ 0.8 were analyzed. *M* molecular mass marker, *1* SisE233S-Hje-C-His, *2* Sis/pSeSD-NPHje-C-His, *3* Sis/pSeSD-Hje-C-His induced by sucrose, *4* Sis/pSeSD-Hje-C-His induced by arabinose. **c** Assessment of upper limit of Hje in SisE233S relative to that in the overexpressed strain induced by arabinose. Whole-cell extracts of Sis/pSeSD-Hje-C-His were diluted at different folds as shown at the *top of the lanes* and separated by SDS-PAGE. *M* molecular mass marker. **d** Hje overexpression resulted in large cells with DNA content with more than two genome equivalents. Flow cytometry profiles showing DNA content and cell size distribution of strains at OD_{600} ~ 0.2. "1C" and "2C" refer to one and two genome equivalents of DNA, respectively

2012), Hje in Δ*hje*/pSeSD-NPHje was anticipated to be elevated 3–5 times compared with that in wild-type (WT) cells. Δ*hje*/pSeSD-Hje, in which Hje was predicted to be highly overexpressed, was more sensitive to HU than Δ*hje*/pSeSD-NPHje, in which Hje was modestly overexpressed. In fact, even in the absence of the DNA-damaging agent, Δ*hje*/pSeSD-NPHje and Δ*hje*/pSeSD-Hje had slower growth. These results demonstrate that overexpression of Hje in the Δ*hje* had a negative effect on cell resistance to HU and on cell growth under normal conditions.

Effects of Hje overexpression on cell growth and morphology

We also observed that overexpression of Hje in SisE233S had similar effects on growth. As shown in Fig. 4a, growth of SisE233S harboring the pSeSD-Hje and pSeSD-Hje-C-His was dramatically reduced in arabinose medium. To confirm that Hje was overexpressed in cells, strains Sis/pSeSD-Hje-C-His and Sis/pSeSD-NPHje-C-His were constructed and expression of Hje-C-His was compared with that in SisE233S-Hje-C-His which encoded C-terminal His-tagged Hje by the chromosomal gene (Fig. 4b). Although the transcriptional level of Hje was elevated 30 times in arabinose medium, according to our microarray data (Table 3), protein was more different. The protein band in SDS-PAGE and Western blot for cells of SisE233S-Hje-C-His and Sis/pSeSD-NPHje-C-His was hardly visible, but the band intensity of Sis/pSeSD-Hje-C-His in arabinose medium was strong and was about 40 times of that in the sucrose medium (Fig. 4b, d). Using serial dilution of samples from Sis/pSeSD-Hje-C-His, we estimated that protein

Table 3 Relative abundance of transcripts of genes related to DNA metabolism, signal transduction, and cell division in the Hje overexpression strain by whole-genome microarray analysis

Process	Gene number (19)	Description	Ratio (pSeSDHje/pSeSD)
Hje	SiRe_0930	Resolvase, Holliday junction-type	29.63
DNA replication	SiRe_0614	DNA polymerase B2 amino-end	3.43
	SiRe_0615	DNA polymerase B2 elongation subunit	2.34
	SiRe_1740	Orc1/cdc6 family replication initiation protein	0.49
Transcription and transcriptional regulator	SiRe_0704	DNA-directed RNA polymerase, subunit M	2.14
	SiRe_0513	Putative transcriptional regulator, AsnC family	2.92
	SiRe_0526	Transcriptional regulator, GntR family	2.18
	SiRe_0553	Putative transcriptional regulator, AsnC family	0.30
	SiRe_2687	Putative transcriptional regulator	2.15
DNA repair	SiRe_0062	Recombination repair enzyme Rad50	2.56
	SiRe_0240	RadC1	2.19
	SiRe_1431	Holliday junction resolvase (Hjc)	0.40
	SiRe_1747	DNA repair and recombination protein RadA	2.48
	SiRe_0565	Conserved hypothetical protein (containing NurA domain)	0.48
	SiRe_0581	AAA ATPase-like protein (containing HerA domain)	0.37
	SiRe_0582	Hypothetical protein (containing NurA domain)	0.25
Kinase	SiRe_2056	Serine/threonine protein kinase	3.25
Cell division	SiRe_0265	ATPase, chromosome partitioning-like protein	0.36

in SisE233S could be less than 1/150 of that in Sis/pSeSD-Hje-C-His induced with arabinose (Fig. 4c). Growth of the SisE233S strain overexpressing Hje-C-His in the liquid medium was also analyzed. Consistent with the plate data, the overexpressing strain had more severe growth inhibition than the control, even more severe than Sis/pSeSD-Hje, in arabinose medium (Fig. 5c and data not shown). Also, flow cytometry revealed that the Hje-C-His-overexpressing strain generated a significant proportion of cells with more than two genome equivalents of DNA (Fig. 4e). Thus, Hje was maintained at an extremely low level in wild-type cells and its overexpression led to reduced cell growth and genome instability.

The phenotype of Hje-C-His overexpression is not due to a metabolic burden of protein synthesis

To confirm that the phenotype of the Hje overexpression strain is Hje dependent, we overexpressed a C-terminal tagged esterase (SisEstA) from *S. islandicus* Rey15A using the pSeSD-SisEstA-C-His vector. In agreement with previous reports (Mei et al. 2012), SisEstA was highly expressed in SisE233S (Fig. 5a, b). Expression of SisEstA was greater than that of Hje-C-His based on band intensity of the two proteins on SDS-PAGE and Western blot (Fig. 5b). However, SisEstA overexpression strain did not reveal any growth defect (Fig. 5c), and did not point to any abnormal DNA content and cell size (Fig. 5d). Thus, overexpression of Hje, rather than a metabolic burden of protein overexpression, produced Hje overexpression strain phenotypes.

Whole-genome microarray analysis of Hje overexpression and deletion strains

To assess the function of Hje in DNA repair, we performed whole-genome microarray analysis of Hje overexpression strain Sis/pSeSD-Hje-C-His. As expected, the transcriptional level of *hje* was increased, about 30-fold, in the overexpression strain (Table 3). Transcription levels of 109 genes were upregulated and 80 genes were downregulated more than twofold (Supplementary Table S2). Most affected genes encode proteins involved in energy metabolism, biosynthesis, and transportation, while some are CRISPR-associated proteins and hypothetical proteins (Supplementary Table S2). Intriguingly, key genes involved in information processing, signal transduction, and cell division were highly represented (Table 3). In particular, two genes encoding DNA polymerase subunits (SiRe_0614 and SiRe_0615) were upregulated by 3.43- and 2.34-fold, respectively. A gene encoding an Orc1/cdc6 family protein (SiRe_1740) was 2.06-fold downregulated. The second group was genes involved in transcription and its regulation, including genes for RNA polymerase (SiRe_0704, 2.14-fold upregulated) and transcriptional regulators SiRe_0513 (2.92-fold upregulated), SiRe_0526 (2.18-fold upregulated), SiRe_2687 (2.15-fold upregulated),

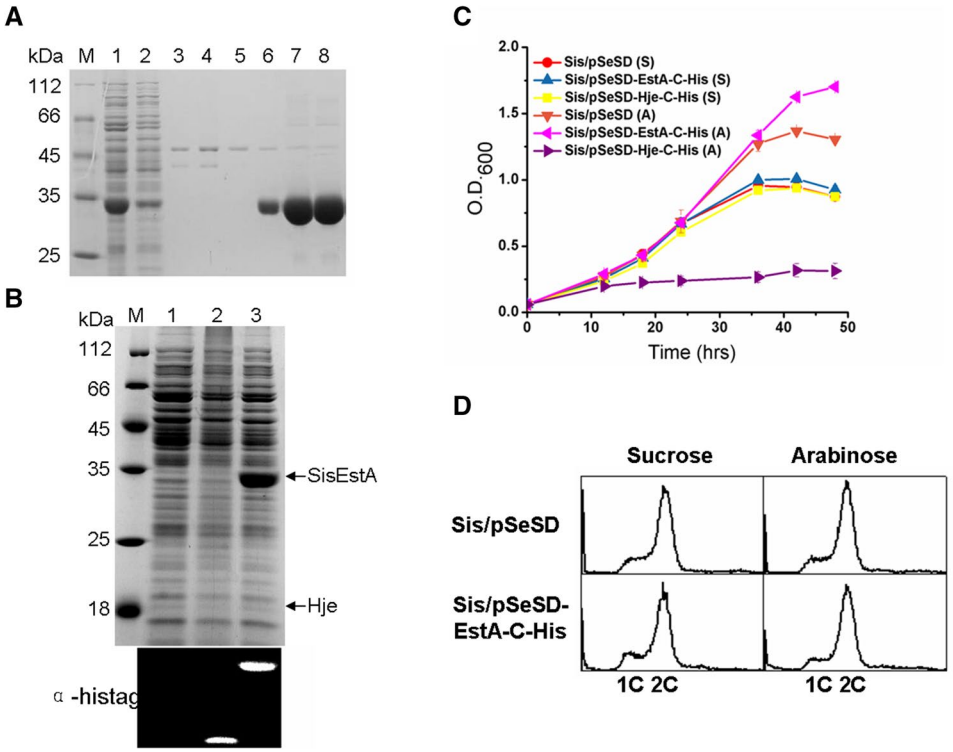

Fig. 5 Strain overexpressing thermophilic esterase did not have phenotypes of the Hje-C-His overexpression strain. **a** SDS-PAGE of samples for overexpression and purification of SisEstA. *M* molecular mass marker, *1* whole-cell extract, *2* flow-through, *3–5* wash fractions, *6–8* elute fractions. **b** Comparison of Hje-C-His and SisEstA-C-His expression by Western blot. *M* molecular mass marker, *1* Sis/pSeSD, *2* Sis/pSeSD-Hje-C-His, *3* Sis/pSeSD-SisEstA-C-His. **c** Growth curves of SisE233S harboring pSeSD, pSeSD-Hje-C-His, and pSeSD-SisEstA-C-His. **d** Flow cytometry showing DNA content of SisE233S harboring pSeSD and pSeSD-SisEstA-C-His at OD_{600} ~ 0.2

and SiRe_0553 (3.33-fold downregulated), respectively. Among the DNA repair genes, those involved (or presumably involved) in recombinational repair, *radA* (SiRe_1747), *rad50* (SiRe_0062), and *radC1* (SiRe_0240) were upregulated by 2.48-, 2.56, and 2.19-fold, respectively, while *hjc* (SiRe_1431) was downregulated by 2.5-fold. Three potential repair genes coding for HerA domain containing protein (SiRe_0581) and NurA domain containing proteins (SiRe_0565 and SiRe_0582) were downregulated by 2.68-, 2.08-, and 3.94-fold, respectively. In addition, a gene for a serine/threonine protein kinase (SiRe_2056) that potentially participates in signal transduction, and a gene for an ATPase (SiRe_0265) that may be involved in chromosome partitioning were upregulated by 3.25-fold and downregulated by 2.78-fold, respectively. The microarray data indicate that Hje must be strictly controlled in cells and its overexpression greatly affected cells fate. Microarray analysis of *hje* deletion stain revealed that only five genes were upregulated and six were downregulated (data not shown).

Discussion

Hje but not Hjc functions primarily in DNA repair

Hjc was the first HJ resolvase characterized in archaea. It is conserved among archaea whereas Hje exists in acidothermophilic crenarchaea including *Sulfolobus* (except *S. tokodaii*), *Acidianus*, and *Metallosphaera*. Genetic analysis in the bacterium *E. coli* indicates that Hjc from thermophilic archaeon *Methanobacterium thermoautotrophicum* promotes DNA repair in resolvase-deficient *ruv* mutants of *E. coli*. Thus, Hjc may be involved in HR and replication fork repair. However, we did not observe a marked growth difference between Δ*hjc* and the WT after genotoxic treatment. Likely Hje-involving responses may provide a primary repair pathway for DSB in the wild-type cells. Hjc may be required under certain circumstances, such as in the absence of Hje or when more DSBs are induced. In agreement with the former concept, *hjc* was downregulated in the Hje overexpression strain (Table 3), suggesting that both Hje and Hjc have a redundant role in HJ resolution.

In SisE233S, Hje and Hjc seem to represent two HJ resolution pathways. We attempted to construct a double mutant of *hje* and *hjc* in SisE233S but failed to obtain a double deletion strain, suggesting a synthetic lethality of both genes. Previous studies of the roles of Hjc from *Haloferax volcanii* and *Thermococcus kodakaraensis* showed that *hjc* in both species produced no obvious phenotype in the presence of DNA-damaging agents (Fujikane et al. 2010; Lestini et al. 2010). However, deletion of both *hef*, a gene encoding a helicase-nuclease (homologous to XPF, a nucleotide excision repair protein in eukarya), and *hjc* resulted in synthetic lethality in *H. volcanii* (Lestini et al. 2010). The study in *H. volcanii* and ours in *S. islandicus* suggest that Holliday junction resolvation is an essential process in both euryarchaea and crenarchaea. Of interest is the role of Hjc

in archaea which do not have another resolvase such as Hje or Hef.

Hje must be maintained at low levels in *S. islandicus*

Hje overexpression resulted in reduced growth rate, genomic instability, and global transcriptomic change. Also, the *hje* deletion strain had reduced DNA repair capacity. Therefore, Hje in the cell must be controlled for effective DNA repair in *S. islandicus*. We measured Hje by Western blot using chromosomally coded Hje-tagged strain by 6 × His, and detected no Hje signal (Fig. 4b). Thus, Hje in the cell is extremely low. Low enzyme content is a typical feature of DNA repair nucleases. Because HJ resolvases play an important role in maintaining genome stability, it is likely that their action must be properly regulated (Fekairi et al. 2009). In addition, it was shown that *S. solfataricus* Hje exhibited much higher cleavage activity than that of Hjc (Middleton et al. 2004; Parker and White 2005). This might be a potential reason why overexpression of Hje was deleterious to cells.

In *S. solfataricus*, the sliding clamp PCNA interacts with Hjc and simultaneously stimulates its HJ resolve activity in vitro (Dorazi et al. 2006). We reported that Hjm (also known as Hel308), a RecQ-like DNA helicase from *S. tokodaii*, can prevent the formation of Hjc/HJ high-order complexes, suggesting a regulatory mechanism of Hjm for Hjc endonuclease activity (Hong et al. 2011). Unlike the regulatory mechanism of Hjc, which involves enzyme activation or inhibition via high-order complex formation and protein–protein interaction, Hje may be controlled by a different mechanism. We speculate that Hje content is controlled by protein post-translation modification. Further study into whether Hje is modified in vivo and how the level and activity of archaeal HJ resolvase Hje are regulated may shed light into the evolution of HJ resolvases and their regulation from prokaryotic to eukaryotic organisms.

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