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Cloning of Genomic DNA Fragment Involved in Acid-Aluminium Tolerance in *Bradyrhizobium japonicum* 38 Through Transposon Mutagenesis

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An acid-aluminium sensitive mutant of *Bradyrhizobium japonicum* 38, designated as AAS38, was generated by mini-Tn*5* transposon mutagenesis. The experiment was carried out to identify acid-aluminium tolerance gene (AAT) in *B. japonicum*. Transposon delivery was carried out through conjugation between *Escherichia coli* S17-1 (λ *pir*) carrying pUTmini-Tn*5*Km1 and acid-Al tolerant *B. japonicum* with different of mating time. Frequency of transconjugation was in the range of 10^{-7}-10^{-6}. A mutant AAS38 was not able to grow on the Ayanaba medium (pH 4.5) containing 50 μM aluminium. However, this mutant formed root nodule of soybean and Siratro plants indicating the gene involved in acid-Al tolerance was not related with nodulation. A 0.8 kb of the genomic DNA fragment flanking the transposon involved in acid-aluminium tolerance was successfully isolated by inverse polymerase chain reaction (Inverse PCR) from AAS38 genome. This fragment was subsequently cloned into pGEM-T Easy (~3 kb) to yield a recombinant plasmid, designated as pGEMT-38 (~3.8 kb), and sequenced. DNA sequence analysis revealed that the genomic DNA fragment had high homology to inner membrane protein from *Salmonella typhimurium* (80% identity and 86% similarity, E-value= 8xe-^{62}) predicted function as efflux transporter.

Key words: *Bradyrhizobium japonicum*, acid-aluminium tolerance, transposon mutagenesis, cloning

Soil condition is a significant factor affecting symbiotic effectiveness between root nodule bacteria (RNB) and leguminous plant. In this mutualism symbiotic, RNB play a critical role to fix N_2 and convert it to NH_3, subsequently assimilated by the host and improves plant growth and productivity. Growth inhibition and lengthened lag phase of RNB have been studied as one of the major effects of high Aluminium (Al) concentration in the acid soil (Keyser & Munns 1979). Richardson *et al.* (1988) have reported that 7.5 μM Al at pH 4.8 could prevent nodulation genes (*nod* genes) expression. Furthermore, Johnson and Wood (1990) have also revealed that PO_4^{3-} is a possible site of action of Al^{3+} in DNA of RNB resulting inhibition of replication DNA.

The slow growing nitrogen-fixing symbiont of soybean, *Bradyrhizobium japonicum,* is among the most agriculturally important plant-associated bacteria due to the ability to supply 50% plant nitrogen demand. Some strains of *B. japonicum* have the ability to grow at Keyser and Munns media (pH 4.5) and tolerance to high Al concentration. The ability to grow at high Al concentration have been studied by Endarini *et al.* (1995) using Ayanaba agar plate (pH 4.5) containing 50 μM Al supplemented to the medium. In addition, to gain high productivity of soybean, physiological response and molecular character information of acid-Al phenotype, it is really important to identify genes which are involves in acid-Al tolerance in *B. japonicum*.

Nowadays, transposon mutagenesis is a successful strategy in order to perform genome-wide identification of essential genes in bacteria (Voleker & Dybvig 1998) and fungi (Firon *et al.* 2003). Transposon are DNA elements which can transpose from one site to another site in the genome (Snyder & Champness 2003). Tn*5* is a major transposon used in mutagenesis of gram negative bacteria (Bruijn & Lupski 1984). Bacterial transposon Tn*5* becomes an important molecular tool because it performs high frequency of transposition, low target sequence specificity and few bacterial genome homologous (Reznikoff 2002).

Transposon mini Tn*5*Km1 was chosen for this research. This transposon is a one of the Tn*5* derivative transposons (de Lorenzo *et al.* 1990) that can randomly transpose to bacterial genome and remain high stability insertion (Herero *et al.* 1990). Insertion of mini-Tn*5*Km1 in bacterial genome resulting kanamycin resistance phenotype. In this research, transposon mutagenesis and inverse polymerase chain reaction (inverse PCR) are major method to identify genome-wide DNA fragment involves in acid-Al tolerance in *B. japonicum*.

In this study, we cloned genomic DNA fragment involved in acid-Aluminium tolerance in indigenous strain of *B. japonicum* through taransposon mutagenesis and analyzed the DNA sequence flanking the transposon.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Growth Media. *B. japonicum* strain 11, 38, and KDR 15 (Endarini *et al.* 1995) were routinely grown on yeast mannitol agar (YMA) media (mannitol 10 g l^{-1}, K_2HPO_4 0.5 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1}, NaCl 0.2 g l^{-1}, yeast extract 5 g l^{-1}) + *Congo Red* (CR) 0.0025%) supplemented with rifampicin (100 μg ml^{-1}) and incubated for 5-7 days at room temperature. *Escherichia coli* DH5α was grown at Luria broth

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(LB) media (tripton 10 g l^{-1}, NaCl 10 g l^{-1}, yeast extract 5 g l^{-1}) or Luria agar (LA) (LB added agar 15 g l^{-1}) and incubated at 37 °C. *E. coli* S17-1 (λ *pir*) carrying pUTmini-Tn*5*Km1 was grown at LB medium supplemented with kanamycin (50 μg ml^{-1}) and ampisilin (50 μg ml^{-1}) (Herrerro *et al.* 1990). Plasmid pGEM-T Easy (~3 kb) (Promega) was used as a cloning vector (TA cloning).

Transposon Mutagenesis. Mutagenesis was carried out using membran filter (0.45 μm) method as described by Wahyudi (1998). Conjugation betweeen cells of the donor strain *E. coli* S17-1 (λ *pir*) harboring pUTmini-Tn*5*Km1 and the recipient strain *B. japonicum* were conducted at 1:1 ratio (approximately 10^8 cells). Conjugation was conducted in LA modification media (composition was the same with LA except NaCl 1 g l^{-1}) and incubated in three different times (12, 18, and 24 hours) at room temperature. After each incubation time has revealed, the mating mixtures were resuspended in NaCl 0.85% and plated on YMA medium supplemented with rifampicin (50 μg ml^{-1}) and kanamycin (50 μg ml^{-1}), and incubated for 7 days at room temperature.

Screening of Acid-Sensitive Mutants. All transconjugant colonies growing on YMA + CR 0.0025% + Rif (50 μg ml^{-1}) + Km (50 μg ml^{-1}) resulted from each *B. japonicum* strain were replicated in the same media. Every each transconjugant colony was screened for acid-Al tolerance using Ayanaba media (pH 4.5) supplemented with Al 50 μM (Ayanaba *et al.* 1983), and incubated for 10 days at room temperature. Transconjugant colonies that were not able to grow on Ayanaba medium (pH 4.5; Al 50 μM) but grew well on YMA + CR 0.0025% + Rif (50 μg ml^{-1}) + Km (50 μg ml^{-1}) were designed as acid-Al sensitive mutants. These mutants were used for further analysis.

Isolation of Genomic DNA from Acid-Al Sensitive Mutant of *B. japonicum*. An acid-Al sensitive mutant of *B. japonicum*, AAS38, was cultured in 25 ml Yeast Mannitol Broth (YMB, YMA without agar) supplemented with rifampicin (50 μg ml^{-1}) + kanamisin (50 μg ml^{-1}) and incubated at rotary shaker (140 rpm) for 4-5 days at room temperature. DNA genome isolation was carried out using the standard protocol as described by Sambrook and Russel (2001).

Isolation of Genomic DNA Flanking Transposon by Inverse PCR. Inverse PCR strategy to isolate genomic DNA flanking transposon of mutant *B. japonicum* genome was conducted using the method as described by Wahyudi *et al.* (2001). DNA genom of mutant *B. japonicum* was digested by *Eco*RV (this enzyme does not digest the transposon). The digested genome was subsequently extracted by phenol/chloroform and ligated (circularized) to initiate a monomeric circle using T4 DNA ligase. Furthermore, the monomeric circle was amplified by 2400 PCR machine (Perkin Elmer, USA) in 50 ml reaction mixture containing 8 μl dNTP 10 mM, 25 μl GC buffer II, 0.5 μl LA Taq Polymerase (Takara, Tokyo, Japan), 1 μl primer Km (I) dan Km (O) at 10 pico mol each, 5 μl circularized DNA and 7.5 μl ddH_2O. Primer sequnces used for the amplification were Km (I): 5'-ACACTGATGAATGTTCCGTTG-3' and Km (O): 5'-ACCTGCAGGCATGCAAGCTTC-3'. The amplification was performed for 30 cycles. Denaturation was conducted at 95 °C for 2 minutes, annealing at 58 °C for 1 minute, elongation at 72 °C for 1 minute and 10 minutes for post PCR phase. Amplified DNA was subsequently separated by 1% agarose gel electrophoresis with 1X TBE buffer.

Cloning of Genomic DNA Fragment Flanking Transposon. The amplified PCR product was purified using the Gene clean III kit (Bio 101) and cloned into the multiple cloning site of pGEMT-Easy (~3 kb; Promega) to construct a recombinant plasmid, designed as pGEMT-38. The recombinant plasmid was subsequently transformed into *E. coli* DH5α by heat shock method (Sambrook & Russel 2001). Transformants were selected on LA plate supplemented with ampicilin (50 μg ml^{-1}) and X-Gal (40 mg ml^{-1}). The recombinant plasmids were extracted from DH5α by standard method (Sambrook & Russell 2001), digested with *Eco*RI, and subsequently separated by 1% agarose gel electrophoresis with 1X TBE buffer.

DNA Sequence Analysis. The recombinant plasmid was isolated from *E. coli* by standard method and used as a template for DNA sequencing. Both strands of genomic DNA flanking the transposon were sequenced using an automatic DNA sequencer ABI 310 (Perkin Elmer, USA) which was conducted at Faculty of Biotechnology, Unika Atmajaya, Jakarta. The universal primers M13 (forward and reverse) were used for cycle sequencing. The sequence was further analyzed by performing homology searches using the BLAST program (Altschul *et al.* 1997) with GenBank and EMBL DNA databases.

RESULTS

Transposon Mutagenesis and Mutant Selection. Transconjugant are recipient cell that the genome has been inserted by transposon mini-Tn*5*Km1 and able to grow in YMA supplemented with kanamycin. This kanamycin resistance was generated by mini-Tn*5*Km1 insertion in the *B. japonicum* genome. Different strains of *B. japonicum* show variability in transconjugant frequency in relation with different mating time. Average transconjugation frequency of three *B. japonicum* strains were in the range of 10^{-6} and 10^{-7} cells per recipients. 24 hours incubation of mating exhibited highest transconjugant frequency for *B. japonicum* strain 11 and KDR 15. In *B. japonicum* strain 38, highest transconjugant frequency was revealed after 18 hours incubation of mating. Frequency transconjugation of mini-Tn*5*Km1 carried by *E. coli* S17-1 (λ *pir*) to *B. japonicum* strains in three different of mating time are shown in Table 1. Two acid-Al sensitive mutants were constructed from *B. japonicum* strain 11, and one acid-Al sensitive mutant were generated from *B. japonicum* strain KDR 15 and 38. Acid-Al sensitive mutants generated from BJ 11 were designated as AAS 11 (1) and AAS 11 (2), otherwise AAS 38 and AAS 15 were designated for acid-Al sensitive mutant generated from *B. japonicum* 38 and KDR 15, respectively. All these acid-Al sensitive mutants were able to form root nodule of siratro (*Macroptilium atropurpureum*) or soybean (*Glycine max*) plant (data not shown). We choosed an acid-Al sensitive mutant, AAS38, for genetic analysis of acid-Al tolerant in *B. japonicum* because it was the first mutant obtained and relatively easy to amplify the DNA fragment flanking the tranposon by inverse PCR from AAS38 genome.

Cloning of DNA Fragment Flanking Transposon. Genomic DNA fragment flanking transposon was amplified by inverse PCR resulting approximately 0.8 kb DNA fragment (Figure 1a). This fragment was succesfully ligated to pGEMT-Easy to generate pGEMT-38 and transformed to *E. coli* DH5α. The recombinant plasmid extracted from these transformants (white colonies) and digested with *Eco*RI resulted ~ 3 kb and ~ 0.8 kb DNA fragment size (Figure 1b). The ~ 3 kb fragment indicated pGEMT cloning vector and the ~ 0.8 kb indicated genomic DNA flanking transposon (insert) which was presumably involved in acid-Al tolerance. Map of this recombinant plasmid, pGEMT-38, is shown in Figure 2.

Tabel 1. Frequency of transconjugation of transposon Mini-Tn5Km1from *Escherichia coli* S17-1 (λ *pir*) to acid-Al *Bradyrhizobium japonicum* with different mating time

Bacterial conjugation	Frequency of transconjugation*		
	12 h	18 h	24 h
B. japonicum KDR 15 x *E. coli* S-17-1 (λ pir)	3.4×10^{-6}	5.1×10^{-6}	6.3×10^{-6}
B. japonicum BJ 11 x *E. coli* S-17-1 (λ pir)	6.7×10^{-7}	1.1×10^{-6}	7.1×10^{-6}
B. japonicum BJ 38 x *E. coli* S-17-1 (λ pir)	6.1×10^{-8}	$6..5 \times 10^{-7}$	1.3×10^{-7}

*Frequency of transconjugation is calculated per recipients

DNA Sequence Analysis. 764 bp long DNA fragment have been sequenced using DNA sequencer ABI 310. Analysis of this sequence employing BLASTX program revealed that it had homology to inner membrane protein from *Salmonella typhimurium* LT2 (AAL22329) (80 identity, 86% similarity, E-value: $8 \times e^{-62}$) encoded by *yhf*K gene (McClelland *et al.* 2001). This inner membrane protein play an impotant role as an efflux transporter. Homology analysis between amino acid sequence of inner membrane protein from *Salmonella typhimurium* and amino acid sequence deduced from sequence of genomic DNA fragment involved in acid-Al tolerance from *B. japonicum* is shown in Figure 3.

DISCUSSION

All strain of *B. japonicum* use in this study have similar morphology. The colony is rounded, white, sticky, slime consistancy and obtain more than one milli litre diametric

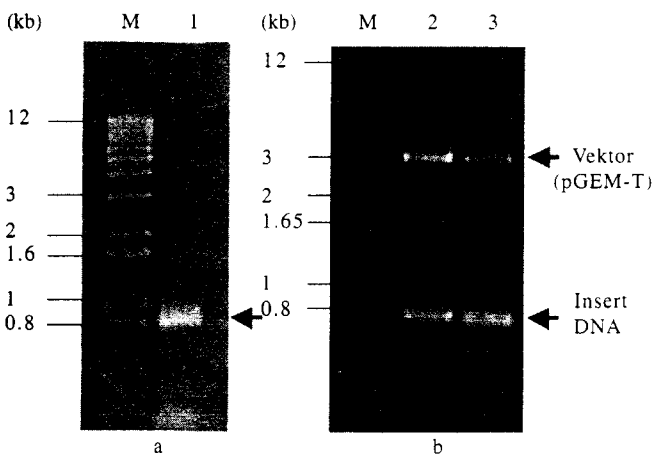

Figure 1. 1% agarose gel electrophoresis of inverse PCR product of genomic DNA flanking transposon (1), recombinant plasmids, pGEMT-38 digested with *Eco*RI (2 & 3), and marker DNA 1 kb ladder plus (M).

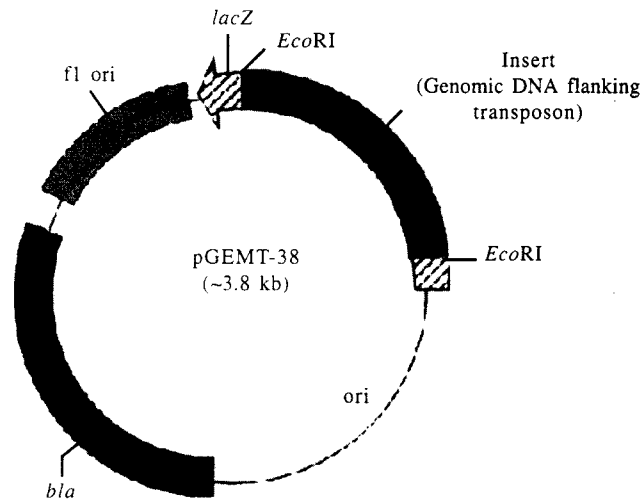

Figure 2. Map of a recombinant plasmid pGEMT-38 (~3.8 kb).

gi|16422024|gb|AAL22329.1| putative inner membrane protein [*Salmonella typhimurium* LT2]
Length=695
Score = 239 bits (610), Expect = 8e-62
Identities = 140/173 (80%), Positives = 150/173 (86%), Gaps = 3/173 (1%)
Frame = -3

```
Query  791  LCLPVAXXXXXXXXXXXXLFSLVPACCNIAGLDTPHKRFFKRLIIGGCLFAGCSLAXFLL  612
            LCLPVAVGL++G L  GLLFSLVPACCNIAGLDTPHKRFFKRLIIG  LFAGCSL   LL
Sbjct  21   LCLPVAVGLLIGQLHLGLLFSLVPACCNIAGLDTPHKRFFKRLIIGASLFAGCSLVTQLL  80

Query  611  LARDIPLPLILTALAMTLGVTAEXXXXXXXXXXXXXXXXIFTLSLAGNLPIWAPLLIYA-  435
            LA  IPLPLILT L + LGVTAEIS LHARLL ASLIA IFTLSLAG +P+W PLLIYA
Sbjct  81   LAESIPLPLILTGLTLVLGVTAEISPLHARLLPASLIAAIFTLSLAGYMPVWEPLLIYAL  140

Query  434  --PWYGLFNWFWFWLWREQPLRESLSVLYRQLADYCEAKYTLLTQHTDPEKSL  282
               WYG+FNWFWFWLWREQPLRESLS+LYR+LADYCEAKY+LLTQH DPEK+L
Sbjct  141  GTLWYGVFNWFWFWLWREQPLRESLSLLYRELADYCEAKYSLLTQHIDPEKAL  193
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Figure 3. Homology analysis between amino acid sequence of inner membrane protein from *Salmonella typhimurium* and amino acid sequence deduced from DNA fragment involved in acid-Al tolerance in *Bradyrhizobium japonicum*.

colony after 10 days of incubation. According to the existence of abundant exopolysaccharides, all *B. japonicum* strain were revealed large watery colony type (Endarini *et al.* 1995). *B. japonicum* has been shown to have the ability to grow at media supplemented with rifampicin 100 μg ml^{-1}, tetracyclin 100 μg ml^{-1} and ampicilin 100 μg ml^{-1}. Moreover, the growth of *B. japonicum* was inhibited by kanamycin 50 μg ml^{-1}. Sensitiveness of kanamycin in *B. japonicum* is one of the reason of using transposon mini-Tn*5*Km1 in this research, which kanamycin performed as a selection marker of transconjugant.

Variability in transconjugation frequency in relation with incubation of mating time may be due to intraspecies genotype diversity. Another critical factor which involves in transposition are DNA polymerase, transcription termination factor (rho factor), and histon like protein (Berg *et al.* 1989) and transposase enzyme activity (Braam *et al.* 1999). Comparison between donor cells and recipient cells concentration in the conjugation mixture has also been reported as a crucial factor affecting transconjugant frequency (Wahyudi *et al.* 1998). However, prolonged of incubation of mating time until 24 h may increased conjugation process and that may have caused more transfer of pUTmini-Tn*5*Km1 from *E. coli* S17-1 (λ *pir*) into *B. japonicum* cell. All mutants sensitive to acid-Al generated from this study were able to form root nodule of siratro or soybean plant (data not shown) indicating genes presumably involved in acid-Al tolerance in *B. japonicum* did not correlate with genes for nodulation.

Approximately 0.8 kb genomic DNA fragment flanking transposon isolated from mutant *B. japonicum* AAS 38 genome was considered involved in acid-Al tolerance. This result might indicates the evidence of genetic regulation for acid-Al tolerance in *B. japonicum* 38. In order to determine the DNA sequence involved in acid-Al tolerance, this ~0.8 kb DNA fragment may be part of the gene involved in the acid-Al tolerance. Studies on the genetic basis of tolerance to low pH suggest that at least two loci of either megaplasmid or chromosomal location of genes that are necessary for the growth of rhizobia at low pH (Chen *et al.* 1991, 1993). However, it is also possible to hypothesized the existence of at least one locus that are responsible to acid-Al tolerance response in *B. japonicum*. Acid tolerance in *R. loti* was related to the composition and structure of the membrane, and expression of one membrane protein of 49.5 kDa and three soluble proteins of 66.0, 85.0, and 44.0 kDa. The expression of these proteins increased when the cells were grown at pH 4.0. (Correa & Borneix 1997). The same authors suggested that acid tolerance in *R. loti* involves constitutive mechanisms, such as permeability of the outer membrane, together with adaptive responses, including the state of bacterial growth and concomitant changes in protein expression. Furthermore, Flis *et al.* (1992) has reported a possible chelation mechanism of exopolysaccharide to cation Al^{3+} as an effort to minimalize Al toxicity in rhizobial cell. Theoretically, those physiological character for acid tolerance may remain genetic regulator system.

Homology analysis of genomic DNA fragment flanking transposon (~0.8 kb) revealed high similarity to inner membrane protein from *Salmonella typhimurium* LT2 encoded by *yhf*K gene (2087 bp). This inner membrane protein plays a role as an efflux transporter. Parra-Lopez *et al.* (1994) has reported the critical role of this protein in antimicrobial resistance and K^+ transport system in *S. typhimurium*. In this study, insertion of mini-Tn*5*Km1 in genomic DNA sequence considered in inner membrane protein coding region resulting interrupted physiological system for *B. japonicum* tolerance acid-Al response. Complex physiological response facing acid-Al stress in the soil was proposed by Glenn and Dilworth (1994). In the presence of high levels of H^+ ions in the environment (less than pH 6), the level of cell membrane permeability was greatly decreased. *B. japonicum* and other rhizobia strictly maintain the internal pH to more basic or several units lower than external pH by H^+ export mechanism and K^+ cytoplasmic accumulation to adapt with acid environment (Aarons & Graham 1991). Furthermore, Glenn and Dilworth (1994) studied another possible mechanisms for acid tolerance response such as alkalinization of external environment, transcriptional activation of inducible protein, transcriptional activation of ATR (Acid Tolerance Response) genes and *act* (acid tolerance) genes.

In *B. japonicum* 38, inner membrane protein might be involved in one of those possible mechanisms. Efflux transporter (pump system) as the major role of the inner membrane protein in *S. typhimurium* may correlate the role of these protein in H^+ export mechanism as an acid responsive mechanism in *B. japonicum* 38. The ability to maintain low internal pH level remain an important mechanism due to effectively minimalize Al^+ intracellular toxicity (Flis *et al.* 1992). Low external pH level may induce proton pump mechanism during electron transport generating a membrane potential that is outside positive. It has been suggested that H^+ efflux may generate to maintain proton gradient. Therefore, inhibition of the proton pumps should lead to a situation where internal pH equal to external pH level caused pH gradient collapse. There are two major classes of proton pumps which are those coupled to respiration and the translocating ATPase. The former can be inhibited by respiratory poisons such as cyanide, and the latter by inhibitors of the ATPase or mutation (Zilberstein *et al.* 1984). Therefore, it is suggested that mini-Tn*5*Km1 insertion caused mutation in proton pumps system of acid-Al tolerance *B. japonicum* 38. Ricillo *et al.* (2000) has analyzed flanking transposon DNA fragment of *R. tropici* and revealed high similarity to *gsh*B gene in *E. coli*. The *gsh*B gene has functioned for glutathione formation and susbsequently used for inhibiting KefB and KefC system, a potassium efflux transpoter found in many bacteria. In addition, it was found that the pH internal (pHi) level in glutathione deficient *E. coli* strain was lower than in the parent strain in medium containing a low level of K^+.

Lower cytoplasmic phosphate level has occasionally revealed in sensitive acid-Al *B. japonicum*, it may due to leak of phosphate uptake mechanism. Phosphate is an important

molecule, because it has the ability to covalently bound Al^{3+} or other toxic Al species, resulting complex molecules considered non-toxic (Flis *et al.* 1992). In addition, to determine physiological acid-Al tolerance response, it may hypothesized the appearance of H^+/phosphate antiport system as one possible mechanism for *B. japonicum*'s surviving strategy in acid-Al environment.

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