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Short communication

A novel gene required for natural competence in *Aggregatibacter actinomycetemcomitans*

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Background and Objective: Natural competence is the ability of bacteria to take up extracellular DNA and incorporate it into their genomes. Some strains of *Aggregatibacter actinomycetemcomitans*, a critical periodontal pathogen, are naturally competent for transformation. However, information on natural competence genes is limited for this species. The aim of this study was to confirm the involvement of a novel gene identified near the fimbriae gene cluster in natural competence.

Material and Methods: The functions of putative open reading frames (ORFs), designated AA00863–AA00865, in the Oralgen project database for *A. actinomycetemcomitans* strain HK1651, have not been determined. Using naturally transformable *A. actinomycetemcomitans* strains D7S-1 and ATCC29523, we created deletion mutants of homologous genes of these ORFs. Natural competence in the study strains was determined using an agar-based transformation frequency assay.

Results: Mutation of the AA00865 homolog, which we named *urpA* in *A. actinomycetemcomitans* strain D7S-1, resulted in the loss of natural competence, whereas mutations of the AA00864 and AA00863 homologs, located downstream of *urpA* gene, did not. Similar results were also observed in the mutants of *A. actinomycetemcomitans* ATCC29523. Complementation of the deleted sequence in the *urpA* mutant restored natural competence.

Conclusion: The *urpA* gene is a novel gene required for natural competence in *A. actinomycetemcomitans* and does not exhibit significant homology with any natural competence genes previously identified in other bacterial species.

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Some bacteria are naturally competent for taking up extracellular DNA and incorporating it into their genome by homologous recombination (1,2). Several hypotheses have been proposed for the function of this natural competence: genome repair, gene acquisition and nutrient acquisition (1,3–8). Based on these putative functions, previous reports suggest that natural competence is advantageous in pathogenic bacteria (9–12). Moreover, a number of genes involved in natural competence have been identified, including the *com* and *pil* genes (2,13). Certain

strains of the gram-negative facultative bacterium, *Aggregatibacter actinomycetemcomitans*, associated with aggressive forms of periodontitis (14), are also naturally competent for transformation and share a transformation system similar to that found in *Haemophilus influenzae* (15,16). In previous studies of *A. actinomycetemcomitans*, a gene cluster (*pilABCD*) encoding type IV pili and a regulatory gene (*tfoX*) were characterized as natural competence genes (16,17). However, information about other natural competence genes is limited for this species.

Fimbria biogenesis genes in *A. actinomycetemcomitans* were found to reside in a 12-kb region containing 14 genes, *flp-1-flp-2-tadV-rcpCAB-tadZABC DEFG* (18,19). Open reading frames (ORFs), designated as AA00863–AA 00865 in the Oralgen project database (http://www.oralgen.lanl.gov/oralgen/), are located upstream of the *flp* operon in *A. actinomycetemcomitans* strain HK1651 and the functions of their products have not been determined. The aim of this study was to examine the role of AA00863–AA00865 homologs in the natural competence of *A. actinomycetemcomitans*. To our knowledge, this is the first study to evaluate the involvement of these ORFs in the natural competence of *A. actinomycetemcomitans* strains. The results led to the identification of a novel competence gene designated as *urpA* (AA00865 homolog).

Table 1. Plasmids and *Aggregatibacter actinomycetemcomitans* strains used in this study

Plasmid/strain	Characteristics	Reference
Plasmid		
pLox2-Spe	Plasmid with a *loxP-spe*^{r}*-loxP* cassette	20
pAT-Cre	Plasmid with a Cre recombinase gene	20
Strain		
ATCC29523	Serotype a, nonfimbriated, natural competence	ATCC
FDA15L	ATCC29523 *Δ*AA00863 replaced with *loxP-spe*^{r}*-loxP*	This study
FDA16ML	FDA15L *Δspe*^{r} gene by pAT-Cre	This study
FDA13L	ATCC29523 *Δ*AA00864 replaced by *loxP-spe*^{r}*-loxP*	This study
FDA14ML	FDA13L *Δspe*^{r} gene by pAT-Cre	This study
FDA11L	ATCC29523 *ΔurpA* replaced with *loxP-spe*^{r}*-loxP*	This study
D7S-1	Serotype a, fimbrial, natural competence	15
FDD23L	D7S-1 *Δ*AA00863 replaced with *loxP-spe*^{r}*-loxP*	This study
FDD24ML	FDD23L *Δspe*^{r} gene by pAT-Cre	This study
FDD21L	D7S-1 *Δ*AA00864 replaced with *loxP-spe*^{r}*-loxP*	This study
FDD22ML	FDD21L *Δspe*^{r} gene by pAT-Cre	This study
FDD17L	D7S-1 *ΔurpA* replaced with *loxP-spe*^{r}*-loxP*	This study
FDD18ML	FDD17L *Δspe*^{r} gene by pAT-Cre	This study
FDD19CL	FDD18ML *ΔloxP* replaced with *loxP-spe*^{r}*-loxP-urpA*	This study
FDD20CML	FDD19CL *Δspe*^{r} gene by pAT-Cre	This study
FL53L	D7S-1 *Δ*upstream of *flp* operon replaced with *loxP-spe*^{r}*-loxP*	This study
LT88	D7S-1 *ΔltxA* gene replaced with the *tet*^{r} gene	This study

Table 2. Primer sequences used for the deletion or complementation of target genes

Primer	Sequence (5′–3′)[a]	Deleted or restored gene
FDpre1	CGAGTGGCATTATAGTCCTG	*urpA*
FDpre2	CCCACGTGGTGCCCATAAATTCATTGCCA	*urpA*
FDpst3	AGCCACGTGGTGTTACACGTAATTCAGACT	*urpA*
FDpst4	ATCGTAAGAAGACTGATCGTC	*urpA*
FDpre5	TGCACGTGGTGTTCCGAAATAGTTTTACGC	*urpA*
FDpre6	TGCATGGCAGTTTGCATTTAC	AA00864
FDpre7	GTCCCACGTGGTGCCCAATTCCGGTAAC	AA00864
FDpst8	CGCCACGTGGTGTACTTTCCTACCCTGAA	AA00864
FDpst9	CAGGCGGCATAAACGGGATTT	AA00864
FDpre10	ATGCTCTCGCTATTGCCGCCG	AA00863
FDpre11	CGCACGTGGTGGTCAATCTTTCGACGCAGT	AA00863
FDpst12	ACCACGTGGTGGGCAACTATGGCTGAAGAA	AA00863
FDpst13	CCCAATCGGGGAAAAAAGTCAC	AA00863
FLpre8	GCGGTAACACGTTTGTCCGA	Upstream of *flp* operon
FLpre9	CACACGTGGTGGTGCATGGCAGTTTGC	Upstream of *flp* operon
FLpst3	GAACACGTGGTGATCGCTATTGCTGTTGC	Upstream of *flp* operon
FLpst4	ACTGAACGGAATAATGGCGAA	Upstream of *flp* operon

[a]Underlined sequences indicate the restriction site of *Dra*III for specific ligation to the *spe*^{r} gene.

Material and methods

Bacterial strains and culture conditions

A. actinomycetemcomitans strains and plasmids used in this study are listed in Table 1. These strains were grown on a modified 3% trypticase soy broth/0.3% yeast extract/5% heat-inactivated horse serum/1.5% agar (sTSB agar) or in 3% trypticase soy broth/0.6% yeast extract (mTSB broth) at 37°C in 5% CO_2 (15,16). When needed, the medium was supplemented with 50 μg/mL of spectinomycin or 6 μg/mL of tetracycline.

Markerless mutations

Target genes in *A. actinomycetemcomitans* strains were deleted with a Cre-*loxP* recombination system, as previously reported (20). Sequences of the primers used for deletion or restoration of target genes by PCR are listed in Table 2. Primer sequences were designed based on the genome information of *A. actinomycetemcomitans* strain HK1651 available from the University of Oklahoma (http://www.genome.ou.edu/). A markerless mutation of the AA00865 homolog in *A. actinomycetemcomitans* strain D7S-1 is shown as an example in Fig. 1A. Briefly, two sets of primers were used to amplify two DNA fragments flanking the target gene. Recognition sites for the restriction enzyme *Dra*III were incorporated into the primers adjoining the target gene and used for ligation between the amplicons and the

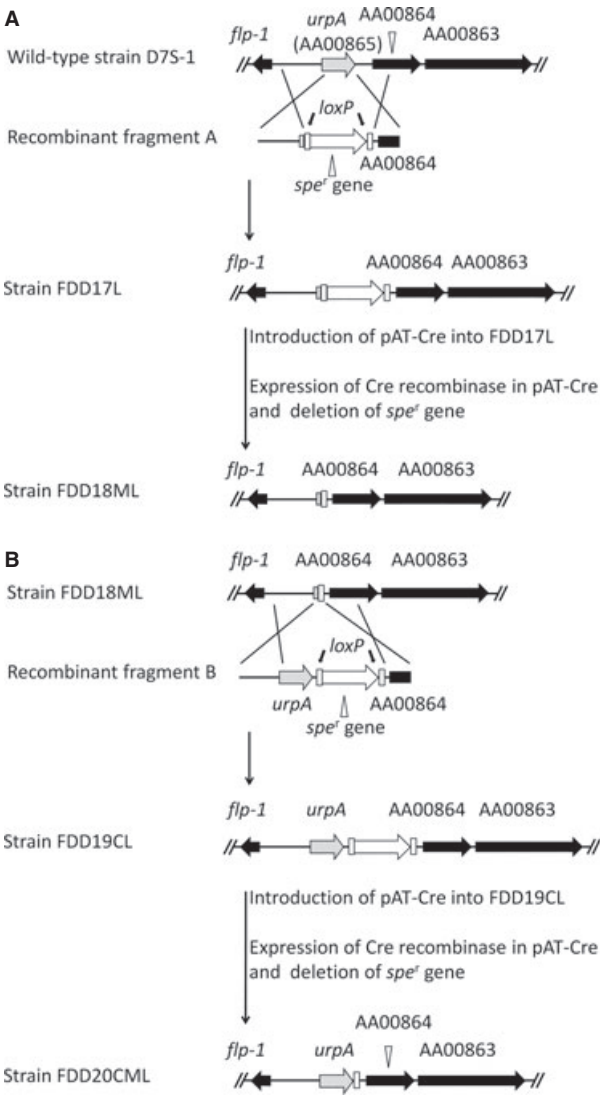

Fig. 1. Markerless mutation procedure for generating a deletion mutant of the *urpA* gene (AA00865 homolog) (A) and its complementation procedure (B). (A) According to the elucidated sequence of the *urpA* gene (gray arrow) located upstream of the *flp–rcp–tad* operon, two DNA fragments flanking the *urpA* gene were amplified and ligated to the *spe*^{r} gene (white arrow) derived from pLox2-Spe (the white rectangles flanking the *spe*^{r} gene are the *loxP* sequence). A constructed fragment (Recombinant fragment A) was used as donor DNA for natural transformation of the D7S-1 strain. The *urpA* gene of the transformant was replaced by the *spe*^{r} gene to generate a mutant, FDD17L. Plasmid pAT-Cre was introduced by electroporation, and recombination at one pair of *loxP* sites eliminated the *spe*^{r} gene. The mutant obtained was named FDD18ML. (B) For complementation of the deleted gene in FDD18ML, the DNA fragment of the *urpA* gene and its upstream region was amplified by PCR using D7S-1 strain genomic DNA. The downstream region of the deleted sequence was also amplified by PCR. Amplicons were ligated to the *spe*^{r} gene derived from pLox2-Spe. The recombinant fragment (Recombinant fragment B) was directly used for the complementation of the markerless mutant FDD18ML through electroporation, and the strain generated was named FDD19CL. Plasmid pAT-Cre was introduced, and recombination at one pair of *loxP* sites eliminated the *spe*^{r} gene. The mutant thus obtained was named FDD20CML.

spectinomycin-resistant (*spe*^{r}) gene (which was flanked by two *loxP* sites) derived from the pLox2-Spe plasmid. The recombinant fragment generated by the *in vitro* ligation was directly used for natural transformation of *A. actinomycetemcomitans* strains. In the transformant obtained, the target gene was replaced by the *spe*^{r} gene flanked by a pair of *loxP* sequences. Some transformants changed to naturally noncompetent. Therefore, plasmid pAT-Cre was then introduced into the primary mutant with or without natural competence by natural transformation or electroporation using an *Escherichia coli* pulser apparatus (18 kV/cm) (Bio-Rad, Hercules, CA, USA), respectively, to eliminate the *spe*^{r} gene via recombination at the *loxP* sites. Finally, a region within the target ORF was replaced with a 34-bp *loxP* sequence for each mutant. PCR analyses with flanking primers suggested that all these colonies had lost the *spe*^{r} gene via recombination at the *loxP* sites.

Restoration of deleted genes in the mutants

In genomic DNA of *A. actinomycetemcomitans* strain HK1651, AA00864 and AA00863 are located consecutively downstream of AA00865. Downstream disruption by the presence of a *loxP* sequence in strain FDD18ML (*Δ*AA00865 homolog) derived from *A. actinomycetemcomitans* strain D7S-1 was examined by the restoration of the deleted gene fragment. The restored strain FDD20CML was generated from a copy of the AA00865 homolog (Fig. 1B). Briefly, the DNA fragment of the deleted region in the AA00865 homolog with its upstream sequence was obtained by PCR (primers FDpre1/FDpre5) using D7S-1 genomic DNA as a template. The downstream region of the deleted region was also amplified by PCR (primers FDpst3/FDpst4). The amplicons were ligated to the *spe*^{r} gene from plasmid pLox2-Spe, as described above. The recombinant fragment generated by the *in vitro* ligation was used to transform the strain FDD18ML by electroporation, and *spe*^{r} transformants were selected. The *spe*^{r} gene in strain FDD19CL was removed by Cre recombinase using pAT-Cre to generate strain FDD20CML, which retained a copy of the *loxP* site after recombination. The

starting point of the downstream sequence next to the *loxP* site in strain FDD20CML was identical to that in the strain FDD18ML.

Natural transformation frequency

Natural transformation assays were performed using an agar-based technique, as described previously (15,16). Briefly, the concentration of recipient bacteria was adjusted with broth to 8×10^9 colony-forming units (CFUs)/mL. A 50-μL aliquot of this bacterial suspension was spotted onto a pre-warmed plate and spread over a small area (10 mm in diameter). After incubation for 2 h, 40 μL of the donor DNA (5 μg/mL) was mixed with the recipient bacteria using a wire loop. The mixture was further incubated for 7 h. Bacterial colonies were picked and plated onto sTSB agar, with and without tetracycline, before being incubated for 4 days to enumerate transformants and total bacteria. Transformation frequency was calculated as the number of transformants per total CFUs. All assays were performed in triplicate. Competence was defined as a transformation frequency of 10^{-7} or higher. Transformation frequency was determined using donor DNA, which was the genomic DNA from a tetracycline-resistant (*tet*^{r}) mutant, LT88, generated from *A. actinomycetemcomitans* strain D7S-1 (Table 1). In a previous study, a deletion mutant of the *ltxA* gene encoding leukotoxin was constructed from a smooth-colony strain, D7S-smooth (21). Genomic DNA of this mutant contains a *tet*^{r} gene as a replacement of the *ltxA* gene, and was used for the construction of *tet*^{r} mutant LT88 from strain D7S-1 by the natural transformation technique in this study.

Aggregation assay

Colonies of *A. actinomycetemcomitans* strain ATCC29523 or its derivative mutant, FDA11L (*Δ*AA00865 homolog), from a plate were inoculated into 3 mL of mTSB broth and cultured overnight. One-hundred microlitres of the first cultured broth was transferred into 3 mL of fresh broth and cultured again in a glass tube (borosilicate glass JR-2; Iwaki, Tokyo, Japan) prepared for the aggregation assay. After 24 h of incubation under static conditions, the optical density of the cultured supernatant ($OD_{supernatants}$) was measured at 590 nm using a spectrophotometer (Colorwave CO7500 Colorimeter; Biochrom Ltd., Cambridge, UK). Aggregated cells on the bottom of the glass tube were dispersed by vigorous vortexing and then the optical density of total cells in the broth (OD_{total}) was measured at 590 nm. The percentage of aggregated cells relative to the total cells was calculated using the following equation: aggregation (%) = $(OD_{total} - OD_{supernatants})/OD_{total} \times 100$. All assays were performed in triplicate.

Results and Discussion

In our preliminary study examining the regulation of fimbriae expression, we noticed that a deletion upstream of the *flp* operon in *A. actinomycetemcomitans* strain D7S-1 rendered the mutant FL53L noncompetent for transformation (Table 3). As the deleted sequence is located within the region between the *flp-1* gene and AA00865 in *A. actinomycetemcomitans* strain HK1651, it can also be part of the promoter sequence for the transcription of AA00865–AA00863. Therefore, this study examined the role of AA00865–AA00863 homologs in the natural competence of *A. actinomycetemcomitans*.

Transformability of generated *A. actinomycetemcomitans* mutants was compared with that of their parental transformable strains D7S-1 and ATCC29523. Distinct differences between these study strains were observed (Table 3). Deletion mutants of the AA00864 homolog (FDA14ML) and the AA00863 homolog (FDA16ML), as well as the parent strain ATCC29523, exhibited a transformation frequency of greater than 1×10^{-5}, which is at least 100 times above the detection limit of the assay. However, nontransformability was found in the deletion mutant of the AA00865 homolog (FDA11L). Similar distinctions were also observed in the transformation experiment using *A. actinomycetemcomitans* strain D7S-1 and its mutants. In the genes analyzed in this study, only the AA00865 homolog was necessary for natural competence of *A. actinomycetemcomitans*. Mutations of the AA00864 and AA00863 homologs, both downstream genes of the AA00865 homolog, did not result in the loss of natural competence. This suggested that the loss of competence in AA00865-deletion mutants was not caused by disruption of the downstream AA00864 and AA00863 homologs. This was further verified by restoration of the deleted sequence in the AA00865 homolog at the original position on the genomic DNA without deletion of the *loxP* sequence in the AA00865 homolog mutant (FDD18ML derived from *A. actinomycetemcomitans* strain D7S-1). The generated mutant, FDD20CML, exhibited a recovered natural-competence phenotype. The presence of the *loxP* sequence upstream of the AA00864 and AA00863 homologs was unlikely to be involved in the loss of natural competence.

In natural competence of gram-negative bacteria, the uptake of exogenous donor DNA occurs from

Table 3. Transformation frequencies of *Aggregatibacter actinomycetemcomitans* strains

Strain	Frequency of natural transformation[a]
ATCC29523	$2.3 \times 10^{-5} \pm 0.8 \times 10^{-5}$
FDA16ML (*Δ*AA00863)	$1.9 \times 10^{-5} \pm 0.6 \times 10^{-5}$
FDA14ML (*Δ*AA00864)	$1.2 \times 10^{-5} \pm 0.1 \times 10^{-5}$
FDA11L (*ΔurpA*)	$< 10^{-7}$
D7S-1	$5.0 \times 10^{-6} \pm 0.2 \times 10^{-6}$
FDD24ML (*Δ*AA00863)	$2.8 \times 10^{-6} \pm 2.6 \times 10^{-6}$
FDD22ML (*Δ*AA00864)	$4.5 \times 10^{-6} \pm 1.7 \times 10^{-6}$
FDD18ML (*ΔurpA*)	$< 10^{-7}$
FDD20CML (Restored *urpA*)	$2.8 \times 10^{-6} \pm 1.2 \times 10^{-6}$
FL53L (*Δ*upstream of *flp* operon)	$< 10^{-7}$

[a]Mean value ± standard deviation.

the cell surface into the cytoplasm (1,2). The double-strand donor DNA binds to the cell surface and is then transferred into the periplasm. One strand is degraded, while the other strand is transported across the cytoplasmic membrane. If the donor DNA has any homologous sequences with genomic DNA, subsequent recombination can lead to transformation of the competent cell via the partial or full incorporation of the donor DNA sequence into the genomic DNA. In theory, the nontransformability observed in the mutants of the AA00865 homolog (Table 3) could be caused by interference of the uptake process or recombination. Our results suggest that the AA00865 homolog is involved in the uptake process of natural competence, based on the following deduction.

The pAT-Cre plasmid, routinely used in our markerless mutations and possessing a *tet*^{r} gene, is functional as an autonomous plasmid in *A. actinomycetemcomitans* and does not possess any homology to the genomic DNA of this bacterial species. This observation was supported by the eventual dilution of the pAT-Cre and the simultaneous loss of resistance to tetracycline during subculture of the plasmid-bearing transformants on plates without antibiotics. Hence, pAT-Cre must have been taken up and repaired to generate an intact circular double-stranded plasmid, rather than being integrated into the genome via recombination. This type of plasmid is therefore useful for evaluating whether the process of DNA uptake in natural competence is intact for the examined strain. A mutant of the AA00865 homolog (strain FDD17L generated from *A. actinomycetemcomitans* strain D7S-1) could not gain pAT-Cre via natural competence (data not shown), demonstrating an interruption of the uptake process in this mutant.

The AA00865 homolog mutant (strain FDA11L) also showed more aggregated growth in broth than the wild-type strain, ATCC29523. In the broth culture, the mutant showed clearer supernatant and a higher percentage of aggregated cells in total cells than the wild-type strain (Fig. 2).

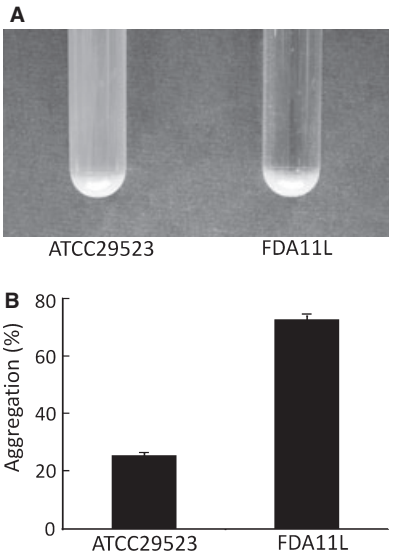

Fig. 2. Aggregations of *Aggregatibacter actinomycetemcomitans* strain ATCC29523 and its derivative mutant, FDA11L (*ΔurpA*), in broth culture. (A) Bacterial cells were cultured in glass tubes for 24 h. (B) The optical density of supernatant cells as well as total cells dispersed by vigorous vortexing was measured at 590 nm, and the percentage of aggregated cells on the bottom of the glass tube in the total cells was calculated. All assays were performed in triplicate.

These results suggest that changes in the cell-surface structure of this mutant had occurred. Furthermore, bioinformatic analyses performed as part of the Oralgen project suggested that this gene encodes a cytoplasmic protein. Combining these results, we suggest that the AA00865 homolog encodes a cytoplasmic protein that is directly or indirectly involved in the cell-surface structure and associated uptake of donor DNA. We named the AA00865 homolog-encoded protein DNA uptake-related protein A (UrpA). For instance, UrpA could be a cytoplasmic protein with a crucial influence on cell-surface structures, such as a gene regulator, a chaperone or a processing enzyme. Further experiments will be needed to demonstrate a relationship of UrpA with DNA-uptake structures on the cell surface.

In addition to the sequence data of *A. actinomycetemcomitans* strain HK1651 from the University of Oklahoma, genomic DNA sequences of *A. actinomycetemcomitans* strains D7S-1 and D11S-1 were also published recently (22,23). Using NCBI BLAST analysis (http://www.ncbi.nlm.nih.gov/), comparing nucleotide or protein sequences and calculating the statistical significance of matches, we compared sequence data of the naturally competent strain D7S-1 with the noncompetent strains HK1651 and D11S-1. The *urpA* gene of strain D7S-1 was 462 nucleotide bases in length and encoded a putative sequence of 154 amino acids. Compared with strains HK1651 and D11S-1, the nucleotide sequence of the *urpA* gene of strain D7S-1 exhibited 99% and 97% identity, respectively. The putative amino acid sequence of strain D7S-1 also showed high identity (98% and 97%, respectively) to sequences of strains HK1651 and D11S-1. In our previous study, the distribution pattern of natural competence in *A. actinomycetemcomitans* strains was found to be concordant with the major clonal lineages of this species (24). However, the *urpA* gene seems to be highly conserved among *A. actinomycetemcomitans* strains, irrespective of the presence or absence of their natural competence. According to bioinformatic analyses performed as part of the Oralgen project, AA00865 of strain HK1651 does not show significant homology with any natural competence genes previously identified in other bacterial species. In our analysis, the *urpA* gene of strain D7S-1 also did not show any significant homology to other gene sequences (data not shown), suggesting that this gene is an entirely novel gene involved in natural competence.

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