Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer

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The capacity of *Campylobacter jejuni* to generate genetic diversity was determined for its flagellar region. Recombination within a genome, as well as recombination after the uptake of exogenous DNA, could be demonstrated. The subunit of the flagellar filament of *C. jejuni* is encoded by two tandem genes, *flaA* and *flaB*, which are highly similar and therefore subject to recombination. A spontaneous recombination within this locus was demonstrated in a bacterial clone containing an antibiotic-resistance gene inserted in *flaA*. A recombinant was isolated in which the antibiotic-resistance gene had been repositioned into *flaB*, indicating that genetic information can be exchanged between the two flagellin genes of *C. jejuni*. The occurrence of recombinational events after the uptake of exogenous DNA by naturally competent bacteria was demonstrated with two mutants containing different antibiotic-resistance markers in their flagellin genes. Double-resistant transformants were formed when purified chromosomal donor DNA was added to a recipient strain, when the two bacterial cultures were mixed under conditions that induce natural competence, or when the two strains were co-cultured. Both mechanisms of recombination may be used by the pathogenic organism to escape the immunological responses of the host or otherwise adapt to the environment.

**Keywords:** flagellin, genetic recombination, horizontal gene transfer

**INTRODUCTION**

The flagella of *Campylobacter jejuni*, a bacterium that causes diarrhoea in humans, are now recognized as a major virulence factor (Newell et al., 1985; Morooka et al., 1985; Black et al., 1988; Aguero-Rosenfeld et al., 1990; Pavlovskis et al., 1991). The flagellar filament consists of flagellin, which is an immunodominant protein during infection (Lane et al., 1987; Nachamkin & Yang, 1992). Two genes encoding flagellin, *flaA* and *flaB*, are present in the genome of *C. jejuni* and *Campylobacter coli* (Nuijten et al., 1990; Guerry et al., 1990; Fischer & Nachamkin, 1991). Expression of the two genes is differentially regulated. Wild-type (WT) bacteria of *C. jejuni* strain 81116 express only *flaA*, and are fully motile, while *flaB* is not expressed. However, bacteria expressing *flaB* have been isolated and these are less motile than WT expressing *flaA* (Wassenaar et al., 1994).

The function of the flagellin B flagella, and of the flagellin gene duplication, is not yet known. It has been suggested that the second flagellin gene may serve as a donor, of which parts would be introduced into *flaA* to compensate for mutations, or to increase the immunogenic repertoire of a given *C. jejuni* strain (Guerry et al., 1992; Tompkins, 1992; Alm et al., 1993), in analogy to the fimbrial gene multiplication of *Neisseria gonorrhoeae* (Meyer, 1987). Two approaches were used to detect such exchange of genetic material between the flagellin genes of *C. jejuni*. First, a mutant of strain 81116 was used, in which a kanamycin-resistance gene (Km<sup>R</sup>) had been inserted in *flaA*. This mutant, R1, has a decreased motility compared to WT, and produces truncated flagella consisting of flagellin B (Wassenaar et al., 1991). Invasiveness of *C. jejuni* for tissue culture cells depends on the presence of flagella and motility. This requirement was used to enrich for motility...
revertants. A revertant was selected from R1 that had regained full motility, and was characterized for DNA recombination between the flagellin genes.

The second approach was based on the fact that C. jejuni is naturally competent to take up extracellular DNA and incorporate this into its genome (Wang & Taylor, 1990; Wassenaat et al., 1993). This horizontal gene transfer may result in the formation of new mosaic flagellin genes. To test this, a second flagellin mutant was constructed, with a tetracycline-resistance gene (Tc\textsuperscript{R}) inserted elsewhere in the flaB gene. The formation of Tc\textsuperscript{R}Km\textsuperscript{R} transformants was tested by incubating chromosomal DNA isolated from the Km\textsuperscript{R} mutant with the Tc\textsuperscript{R} mutant as a recipient strain and vice versa. Moreover, the formation of double transformants was determined in mixtures of the single mutants under conditions that favour natural transformation and after co-cultivation of the single mutants on the same plate.

METHODS

**Bacterial strains and growth conditions.** The Escherichia coli strain used for cloning and vector isolation was PC2495 [\textit{thi} \textit{dellac-proAB proteR supE recA56 F'(proAB' lacIqZ- AdelM tralD36)] (Phabagen Collection, Department of Molecular Cell Biology, University of Utrecht). The origin of the C. jejuni strain 81116 and the flagellin mutants R1 (\textit{flaA}: Km\textsuperscript{R}) and R3 (\textit{flaB}: Km\textsuperscript{R}) of this strain has been described elsewhere (Wassenaat et al., 1991). MPI (\textit{flaB}: Km\textsuperscript{R}) is a motility phenotype revertant derived from R1 (this study). T1 (\textit{flaB}: Tc\textsuperscript{R}) is a mutant of 81116 containing a Tc\textsuperscript{R} gene inserted in its \textit{flaB} gene and is derived from 81116 by electroporation with plVB: \textit{flaB}: \textit{TCa}, a Bluescript vector which contains \textit{flaB} sequences interrupted by a Tc\textsuperscript{R} gene (see below). All strains were cultured at 42 °C on saponin plates (Nuijten et al., 1989) supplemented with 30 µg kanamycin ml\textsuperscript{-1}, or 20 µg tetracycline ml\textsuperscript{-1}, when appropriate, under microaerobic conditions. Bacterial motility was confirmed by colony size on thiglycollate plates containing 0.4% agar (Caldwell et al., 1985).

**Construction of plVB: \textit{flaB}: \textit{TCa}.** The Tc\textsuperscript{R} gene that was inserted into \textit{flaB} was originally derived from a WT Campyllobacter vector. It was isolated from pUOA15 (Wang & Taylor, 1990) by partial Sau3A digestion. A 5 kb fragment conferring Tc\textsuperscript{R} to \textit{E. coli} was cloned into the BamHI site of pBluescript II KS(+) (Stratagene). This insert was then introduced into the Ndel site located in the 3'-end of the \textit{flaB} gene present on plVB3-300 (Nuijten et al., 1990) by blunt-end ligation after filling in the sticky ends of the restriction fragments with DNA polymerase.

**Electroporation of C. jejuni 81116 with plVB: \textit{flaB}: \textit{TCa}**. C. jejuni 81116 was cultured on Mueller-Hinton (MH) agar plates and electroporation was induced as described by Wassenaat et al. (1993). Vector DNA plVB: \textit{flaB}: \textit{TCa} was isolated from \textit{E. coli} PC2495 by the alkaline lysis procedure and purified by CaCl\textsubscript{2} density centrifugation (Sambrook et al., 1989). A sample of 5 µg DNA was transformed into 10\textsuperscript{6} electrocompetent bacteria according to Wassenaat et al. (1991) with one modification to increase the yield of transformants: after the pulse the bacteria were left to recover on MH agar plates for 24 h in a microaerobic atmosphere before they were harvested and plated out on Tc selective saponin plates. This resulted in seven Tc\textsuperscript{R} transformants. Chromosomal DNA was isolated from five transformants, digested with EcoRV and hybridized with the 5 kb Tc specific fragment of pUOA15 and with the flagellin specific insert of plVB3-300. A mutant, T1, was selected which contains the Tc insert in its \textit{flaB} gene, as was concluded from the hybridization (results not shown).

**Invasion of R1 into tissue cultured cells.** Bacterial invasion into INT-407 cells was performed as described elsewhere (Wassenaat et al., 1991). Briefly, intestinal cells 407 were cultured to a confluent monolayer (~10\textsuperscript{6} cells per well) and loaded with a bacterial suspension (~10\textsuperscript{9} bacteria) of R1. Bacteria were allowed to invade the cells for 30 min at 37 °C in 5% CO\textsubscript{2}, 95% air. After the monolayer was washed five times, it was incubated with medium containing 250 µg gentamycin ml\textsuperscript{-1}. This treatment protects internalized bacteria only, as the antibiotic does not enter the cells. After washing, the cells but not the bacteria were lysed with 0.5% Triton X-100. Internalized bacteria were recovered by culturing on saponin plates. The motility revertant MPI of R1 was selected from recovered bacteria by the swarming appearance of the colony on saponin plates. Motility was confirmed on thiglycollate agar plates.

**Western blot analysis.** Western blot analysis of whole bacterial protein extracts was performed as described by Wassenaat et al. (1991). Monoclonal antibodies (mAbs) CFI and CF17, which were raised against flagellin of C. jejuni 81116 (Newell, 1986), were used in dilutions of 1:500.

**PCR analysis.** PCR reactions were performed on single C. jejuni colonies. A toothpick stab of cells was suspended in 100 µl 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, and 5 µl was used in a 100 µl reaction mix for amplification. The following primers were used: primer A1 (\textit{6'-CATATGAAACGACTAAAAG-3'}) is located upstream of \textit{flaA} at position -317 to -300 (the initiating ATG-codon starts at +1); primer B1 (\textit{5'-TTAATGTTAAAGCAGCACG-3'}) is located within the coding region of \textit{flaA} at nucleotide position +1576 to +1593 (corresponding with position -329 to -312 of \textit{flaB}); and primer C (\textit{5'-ACCAACAGCTATATCTCCC-3'}) is complementary to both flagellin genes, at position +931 to +948. PCR reactions were performed using Taq DNA polymerase (Promega) with 35 cycles of 1 min at 90 °C, 2 min at 45 °C and 3 min at 72 °C.

**Sequence analysis.** The partial nucleotide sequence of the PCR products was determined by dyeoxy sequencing using T7 DNA polymerase (Pharmacia sequencing kit) and 5 µCi [\textit{32P}]dATP (185 kbq; 3000 Ci mmol\textsuperscript{-1}). Prior to sequencing the PCR products were excised from agarose gel and purified using Gene-Clean (Bio-101). Purified PCR product (1 µg) was de-natured by incubation with NaOH and precipitated with ethanol. For the sequence reaction the PCR primer C and two nested primers were used: primer A2 (\textit{5'-CTTATGACGAGCTATCG-3'}) is identical to position +10 to +36 of \textit{flaA} and nested primer B2 (\textit{5'-AGGATACACGACACGATC-3'}) is identical to nucleotides +10 to +36 of \textit{flaB}.

**Natural transformation.** For natural transformation of C. jejuni 81116, mutant R1 and mutant T1 with Campylobacter chromosomal DNA, the acceptor strain was cultured overnight on saponin plates, harvested in MH broth and diluted in MH broth to a suspension of 10\textsuperscript{8} c.f.u. ml\textsuperscript{-1}. This suspension was incubated at 37 °C for 3 h under microaerophilic conditions to induce competence. Transformation was carried out in biphasic medium in a capped and pierced microcentrifuge tube containing 1 ml MH agar which was overlaid with 200 µl competent cells. Chromosomal DNA (10 µg dissolved in water) was added and incubated for 2 h at 37 °C under microaerophilic conditions. Transformation was terminated by the addition of 50 µg DNase I.
(Bovine Pancreas DNase I, grade II; Boehringer). In the controls 50 μg DNase was added prior to the DNA. Cells were harvested and plated out on saponin plates supplemented with the selective antibiotic to determine the number of transformants.

For transformation in mixed cultures, bacteria of each strain were cultured, harvested and diluted in MH broth as described above. Equal amounts (200 μl each) of each strain were mixed and overlaid on 1 ml MH agar in a microcentrifuge tube as above. In the controls, cells of each strain were diluted in PBS (0.145 M NaCl; 0.15 M sodium phosphate) and mixed together in equal amounts. When indicated, 50 μg DNase I was added to the 400 μl cell suspension. The mixture was left for 5 h at 37 °C under microaerobic conditions, and dilutions were plated on selective saponin plates to determine the number of transformants.

To monitor natural transformation during co-cultivation, T1 and R1 were grown on saponin plates separately and equal amounts of bacterial suspensions (about 10^4 c.f.u.) were mixed and cultured on saponin plates for 48 h. After this incubation, the bacterial lawn was harvested in 1 ml PBS and dilutions were again plated out. The number of T1 and R1 was determined on plates containing Tc and Km, respectively, and the number of double-resistant transformants on plates containing Tc and Km.

RESULTS

Intra-genomic recombinations between flaA and flaB

Motile bacteria containing an active flaA gene are more invasive for tissue culture cells than non-motile bacteria. This property was used in an invasion assay to select fully motile variants originating from the non-motile mutant R1, which contains a KmR gene inserted into its flaA gene. Of the 3 × 10^2 c.f.u. recovered from the cells after invasion, one colony had the swarming appearance on saponin plates typical for WT. The colony shape of this clone was also similar to WT in thioglycollate plates (results not shown). However, these motile bacteria, designated MP1 for motility phenotype 1, were still kanamycin resistant.

The flagellin content of MP1 was analysed on Western blots, using mAbs CF1 and CF17. mAb CF17 binds to a conserved epitope present on flagellins A and B, whereas mAb CF1 recognizes an epitope which is present on flagellin A only, situated between amino acids 259 and 275 (nt 775–825) (Nuijten et al., 1991). The parental strain R1 and the derived MP1 mutant produce flagellin that is recognized by monoclonal CF17 but not by CF1 (Fig. 1). WT and mutant R3, which were included as controls, produce flagellin that can be detected with both CF17 and CF1. Mutant R3 contains a KmR insertion in flaB and is fully motile by means of flagellin A flagella (Wassenaar et al., 1991). These results show that the flagellin protein of MP1 lacks the flagellin-A-specific epitope recognized by mAb CF1.

To obtain information on the organization of the flagellin genes of MP1, PCR analysis was performed on the genomic DNA, with flaA- and flaB-specific oligonucleotides as primers. As expected, the flaA-specific primer set A1 + C resulted in an extended PCR product III with R1 compared to fragment I of WT, due to the KmR insertion. In contrast, these primers resulted in a PCR product I of MP1 that was identical in length to that of WT (Fig. 2). Conversely, the PCR product IV of MP1 obtained with the flaB-specific primer set B1 + C was identical in length to the KmR-containing flaA product III of R1. These results indicate that the KmR gene of MP1 is inserted in flaB. The lack of binding of mAb CF1 to flagellin of MP1 suggests that during a recombinational event in R1, which led to MP1, flaA sequences had been removed from their original context.

The PCR fragments obtained from MP1 (Fig. 2) were used to determine the nucleotide sequences of the 5'-end of the flagellin genes where flaA and flaB are different and can be distinguished (Nuijten et al., 1990; GenBank/EMBL Data Bank accession number J05635) (Fig. 3). The results show that the recombination event upstream of the KmR gene had occurred between nt 90 and nt 156, since in this region the DNA sequence of MP1 changes from flaA to flaB as compared to WT. The recombination event downstream of the KmR gene should have occurred downstream of nt 825, positioning the CF1-binding epitope in the second, interrupted, gene of MP1. This epitope is not expressed in MP1 (Fig. 2). The site of recombination downstream of the KmR gene could further be deduced from the size of the PCR product obtained with primers B1 and C on MP1. Primer B1 is situated within the coding region of the WT flaA at nt 1576. This part of flaA seems to be conserved in MP1. Thus, the recombination site has occurred between nt 825 (after the CF1 epitope) and 1576. However, the exact position of this cross-over event could not be determined, since the flagellin genes of C. jejuni 81116 are identical between nt 825 and 1527.

Two different recombinational events could theoretically result in the formation of MP1 (Fig. 3): two double cross-over recombinations within the genome of R1 (intra-genomic recombination) or four independent single cross-overs between two genomes (inter-genomic recombinations). In our view MP1 originated from an intra-genomic recombination. The second model allows for duplications of flaA-specific sequences that would replace flaB-sequences without being lost in their original position, or vice versa. Such duplications are not present within the distinguishable 5' part of the fla genes. It is very unlikely that the four independent single cross-overs of the second model have occurred at homologous positions. A more likely origin of MP1 is an intra-genomic recombination with two double cross-overs in the R1 genome. However, as shown below, inter-genomic recombinations do occur in the flagellin locus of C. jejuni.

Horizontal gene transfer

To determine whether genetic exchange between flagellin sequences does occur between members of a bacterial population, a second antibiotic-resistance gene was introduced into the flagellin region of C. jejuni 81116. This TcR gene, isolated from a natural Campylobacter plasmid, was cloned into the 3'-region of flaB, whereas the KmR gene in R1 had been inserted in the 5'-region of flaA. The two
mutants T1 and R1 were used to monitor the formation of double-resistant mutants of C. jejuni.

In the first set of experiments, 10 μg chromosomal DNA of T1 was mixed with competent R1 bacteria under conditions that allow transformation to occur in WT (Wassenaar et al., 1993). Transformants were selected on plates containing Km and Tc. By analogy, R1-derived chromosomal DNA was mixed with competent T1 cells. As a control, competent WT cells were transformed with DNA of T1 or R1, and in this case transformants were selected on Tc and Km plates, respectively. The results show that T1 DNA can indeed transform R1 and vice versa, with a frequency slightly below that of WT (Table 1). The addition of DNase together with DNA largely prevented the formation of double-resistant transformants; the omission of donor DNA totally prevented their formation, indicating that they arise from the transfer of DNA. Southern blots of genomic DNA of the double-resistant transformants hybridized with probes specific for the Km or Tc cassette confirmed the presence of both markers in the flagellin locus of these transformants (results not shown).

Double-resistant mutants were also formed when cultures of T1 and R1 were mixed in a biphasic medium, which permits natural transformation (Wang & Taylor, 1990). In contrast, mixed cultures suspended in PBS, which does not allow natural transformation, did not result in any detectable double-resistant mutants. Natural transformation in biphasic MH medium was determined with 8 × 10^7 c.f.u. R1 mixed with 8 × 10^7 c.f.u. T1, which resulted after 5 h in 1.2 × 10^-5 Tc^R Km^R mutants per total c.f.u. The formation of Tc^R Km^R mutants is 1000-fold
FIG. 3. (a) Part of the nucleotide sequence of the flagellin genes of WT (GenBank/EMBL Data bank accession number J05635), R1 and MP1 from nt 1 to nt 90 (amino acids 1-30) and nt 157–nt 180 (amino acids 52–59). The nucleotide sequences of flaA and flaB are identical between nt 91 and 156 and are not shown. flaB sequences are given in italics. The KmR insert (lower case) of R1 was inserted in the BglII site (underlined in WT). The obtained sequence of MP1 suggests that the cross-over event upstream of the KmR gene had taken place between nt 90 and 156. (b) Two possible recombinational events can result in the formation of MP1: an intra-genomic recombination, requiring two double cross-overs within the genome of R1 (top); or inter-genomic recombinations, requiring four single cross-overs between two genomic molecules (bottom). For clarity, flaA, flaB and the intergenic region are not drawn to scale. Note that the donor DNA of R1 in the lower part of the figure is drawn from right to left. The direction of transcription is indicated by arrows.

Table 1. Transformation of naturally competent cells with isolated DNA

<table>
<thead>
<tr>
<th>Acceptor strain</th>
<th>Donor DNA</th>
<th>No. of transformants per no. of acceptor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>T1</td>
<td>7.3 x 10^5</td>
</tr>
<tr>
<td>WT</td>
<td>T1</td>
<td>2.0 x 10^4</td>
</tr>
<tr>
<td>T1</td>
<td>R1</td>
<td>6.0 x 10^5</td>
</tr>
<tr>
<td>WT</td>
<td>R1</td>
<td>4.3 x 10^4</td>
</tr>
<tr>
<td>T1*</td>
<td>R1</td>
<td>1.4 x 10^7</td>
</tr>
</tbody>
</table>

*DNase I was added prior to the addition of DNA.

Finally, it was tested whether transformation also occurs when TcR and KmR mutants are cultivated together. The two mutants were mixed together in equal quantities (6.3 x 10^8 c.f.u. T1 and 2.5 x 10^9 c.f.u. R1) and co-cultivated on saponin plates for 48 h. The bacteria were then harvested, diluted and replated on three types of saponin plates: plates containing Tc resulted in 4 x 10^6 c.f.u. T1; plates containing Km resulted in 4 x 10^6 c.f.u. R1; and plates containing Tc and Km resulted in 1 x 10^9 c.f.u. newly formed double-resistant mutants. The addition of 50 μg DNase to the cell suspension prior to plating and cultivation decreased the number of formed TcR-KmR mutants to 3 c.f.u. These results show that during conditions of growth, the frequency of genetic transfer between mutants derived from the same strain is about 1 x 10^-7 mutants per total c.f.u.

DISCUSSION

A motility revertant of flagellin mutant R1, MP1, was selected after invasion of bacteria into tissue cultured cells. MP1 originated from R1 by a spontaneous recombination of the flagellin genes, which had repositioned the KmR gene insert into the downstream flagellin gene. Recently, spontaneous recombinations occurring in the flagellin region were also described for C. coli (Alm et al., 1993), which has a flagellin gene organization similar to C. jejuni 81116. In this case, a single intra-genomic cross-over resulted in a deletion mutant expressing a chimerical, functional flagellin protein, composed of flagellin A and B parts. Moreover, from an R1-like flaA flaB* C. coli mutant motile KmR transformants were isolated that were lowered by the addition of 50 μg DNase I, which resulted in 2.5 x 10^-8 transformants per total c.f.u. This suggests that the mutants were the result of transformation, rather than conjugation. The number of transformants obtained in mixed cultures is a factor of 5–6 lower than obtained after transformation with 10 μg DNA (Table 1), which may be a reflection of the amount of free DNA present in the mixed cultures.
ascribed to inter-genomic recombinations after natural transformation, removing the Km\textsuperscript{R} gene from flaA and inserting a new Km\textsuperscript{R} gene in flaB (Alm et al., 1993), as depicted in the lower half of Fig. 3(b). Although such an event could also explain the formation of MP1, we favour the simpler explanation of two double cross-over recombinations within one genome (top half of Fig. 3b), for the following reason: no duplications of specific flaA or flaB sequences were found in the flagellin locus of MP1. This indicates that the two cross-over events took place in the same position in the two genes (the exact positions could not be determined due to the strong homology of the genes, see Fig. 2). It is unlikely that this occurs in four independent single cross-overs between two genomes; two double cross-over recombinations within one genome does not allow sequence duplications.

The occurrence of recombinations between the duplicated flagellin genes of C. jejuni can be of importance for its virulence: variation in the immunogenic properties of flagella may be an advantage for the bacteria to evade the host’s immune response, as is shown for other organisms (recently reviewed by Brunham et al., 1993). The best studied example is the antigenic variation of pili (fimbriae) of N. gonorrhoeae. Pilin variation involves the re-arrangement of a partial pil gene from one of the many silent loci into an expression locus (Gibbs et al., 1989; Meyer et al., 1990). In comparison, the flagellin variation of C. jejuni is limited as there are only two flagellin genes present. Another difference is that the silent pil genes of N. gonorrhoeae do not contain promoter sequences and are incomplete. In contrast, the complete flaB gene of C. jejuni is preceded by promoter sequences and can be expressed under certain circumstances (Wassenaar et al., 1994).

Bacteria expressing flagellin B flagella are less motile, and are less able to invade tissue culture cells in vitro (Wassenaar et al., 1994). Therefore, the two flagellin genes present in C. jejuni are probably not alternately expressed during infection, when motility is important. However, as is shown in this study, the incorporation of epitopes of flagellin B into flagellin A can result in fully motile flagella with altered antigenic properties. If such epitopes are surface exposed along the flagellar filament, as was suggested by Nuijten et al. (1990), and if the immune response reacts selectively to flagella, the formation of such recombinants in vivo would be advantageous for the bacteria.

A second mechanism to produce antigenic variation in bacteria is the uptake of DNA by natural transformation followed by recombination. Natural transformation is also described for N. gonorrhoeae, and causes fimbral variation at higher frequencies than intra-genomic recombinations (Seifert et al., 1988; Meyer et al., 1990). In this case, the uptake of DNA is dependent on specific sequences (Goodman & Scocca, 1988). This paper describes the occurrence of recombinations between the DNA of two C. jejuni mutants after natural transformation. Two antibiotic-resistance markers were introduced into the flagellin locus to monitor such recombinations. The results indicate that transfer of DNA is very efficient between mutants derived from the same C. jejuni strain. It has been suggested that specific sequences are required for DNA uptake during experimentally induced natural transformation (Taylor, 1992). Although transfer of DNA between different strains or species is less efficient, it does occur (Wassenaar et al., 1993). The presence of purified DNA is not required for transformation, as a high number of transformants were obtained from two mutants during co-culturing under laboratory conditions. The frequency of genetic transfer under environmental conditions needs to be determined, but, in analysing Campylobacter strains arising from multiple strain infections, the possibility of genetic transfer must be taken into account.

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