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Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon

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We have tested the importance of genetic variation in the major histocompatibility complex (MHC) class IIB in Atlantic salmon (*Salmo salar*) for survival after challenge with a highly virulent bacterial pathogen. Forty juvenile full siblings from each of 120 families were infected with the bacterium *Aeromonas salmonicida*, which causes high mortality in salmon due to furunculosis. Fishes from high-resistance (HR, < 35% mortality) and low-resistance (LR, > 80% mortality) families were screened for their MHC class IIB genotypes using the denaturing gradient gel electrophoresis (DGGE) technique. The exon 2 sequences, encoding the major part of the peptide-binding region, were established for each DGGE fragment. One allele, *e*, containing a missense single base substitution was significantly more prevalent in HR families than in LR families. An odds-ratio test showed that broods carrying this allele had a 12-fold higher chance of being HR than broods without the *e* allele. A second allele, *i*, showed significantly higher frequencies in uninfected and surviving individuals than in infected dead individuals. A third allele, *j*, tended to be more prevalent both in LR families and in individuals that had died of the infection. There was no correlation between MHC heterozygosity and resistance to *A. salmonicida*. Our results support the hypothesis that MHC polymorphism is maintained through pathogen-driven selection acting by means of frequency-dependent selection rather than heterozygous advantage.

Keywords: major histocompatibility complex class II; bacterial infection; alleles; Atlantic salmon; experiment

1. INTRODUCTION

The genes in the major histocompatibility complex (MHC) are crucial for the presentation of self peptides and foreign-antigen peptides to the T-cells in the adaptive immune system. Both MHC class I and class II, in mammals, consist of several distinct loci, which are represented by multiple alleles with strikingly uniform distribution within populations (Klein 1990). How this extreme level of genetic variation is maintained is strongly debated (Potts & Slev 1995; Apanius *et al.* 1997; Edwards & Hedrick 1998; Paterson *et al.* 1998). Several studies of DNA sequence variation demonstrate convincingly that some sort of positive balancing selection is acting on the MHC (Hughes & Nei 1989; Klein & O'hUigin 1994). It has been assumed that pathogens are the most important agent behind the balancing selection (Klein 1986; Klein & O'hUigin 1994). Complementary mechanisms to pathogen-driven selection are disassortative mating acting through olfactory-based mate choice and, in mammals, maternal foetal selection (Potts *et al.* 1991; Wedekind *et al.* 1995; Edwards & Hedrick 1998; Penn & Potts 1999).

Parasite-driven selection and sexual selection can be operating at the genetic level in at least two ways: first, by heterozygous advantage (Hughes & Nei 1989) and second, by frequency-dependent selection (Slade & McCallum

1992). Heterozygous individuals are assumed to detect and present a wider range of pathogen-derived antigens due to a larger number of different MHC molecules. The few cases indicating heterozygous advantage include associations between MHC heterozygosity and the development of AIDS from HIV infection, and MHC class II heterozygosity and hepatitis B viral infection (Thursz *et al.* 1997; Carrington *et al.* 1999). The alternative mechanism is negative frequency-dependent selection, where rare alleles have a selective advantage over common alleles. More studies support this latter mechanism in terms of correlations between certain MHC genotypes or MHC alleles and disease resistance or other fitness traits (Briles *et al.* 1983; Hill *et al.* 1991; Slade & McCallum 1992; Von Schantz *et al.* 1996; Paterson *et al.* 1998).

We have studied the effects of MHC heterozygosity and MHC alleles on resistance to furunculosis caused by the Gram-negative proteobacteria *Aeromonas salmonicida* in Atlantic salmon. This bacterium was introduced to Norway following import of salmon from Scotland in 1985 (Johnsen & Jensen 1994). Since then, it has spread to a large number of hatchery populations of Atlantic salmon and brown trout (*Salmo trutta*), which subsequently transmit the disease to wild populations (Johnsen & Jensen 1994). There is a high heritability of mortality to furunculosis ($h^2 = 0.48$) and selection experiments have produced lines with high resistance (Gjedrem *et al.* 1991; Gjedrem & Gjøen 1995). Previous results show that Atlantic salmon in the Baltic Sea have a single MHC

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Table 1. Numbers of high resistance (HR, < 35% mortality when infected with the bacterium *Aeromonas salmonicida*) and low resistance (LR, > 80% mortality) broods of Atlantic salmon from which dead, surviving and uninfected individuals were sampled

(Numbers of sampled individuals per brood are also shown (individuals per brood). Dead and surviving individuals had taken part in the challenge test, while the uninfected individuals were their full-sibs that had not been exposed to the pathogen.)

	dead		surviving		uninfected	
	broods	individuals per brood	broods	individuals per brood	brood	individuals per brood
HR	5	5	4	2–4	5	5
LR	6	4–5	1	3	5	5
total	11	54	5	16	10	50

class IIB locus with up to 16 alleles within populations (Langefors *et al.* 1998, 2000). The aims of the present study on Atlantic salmon were to examine the importance of MHC class II genes for resistance to an infectious disease, to investigate whether pathogen-driven selection is of importance for MHC variation and, if so, what selective mechanisms are acting on the MHC in the presence of a pathogen. Using denaturing gradient gel electrophoresis (DGGE) and sequence analysis, we have focused on exon 2 of the MHC class IIB gene, which encodes the major part of the peptide-binding region of the MHC class II molecule (Brown *et al.* 1993).

2. MATERIAL AND METHODS

(a) Challenge test and samples

At AkvaGen, Sunndalsøra, on the west coast of Norway, 120 full-sib families were tested for factors including growth rate, sexual maturation, coloration, fat content and disease resistance each year. The aim of these tests was to produce strains with high 'breeding values', i.e. high values of the aforementioned traits, for commercial farming of salmon. The test fishes belonged to a population that originates from a number of wild Norwegian populations and has been maintained under farming conditions for six generations. Resistance to furunculosis is one of the traits that the fishes have been tested for annually since 1991.

For the challenge test in 1996, 40 juveniles (one year old) from each of the 120 families were sampled at random at the breeding station in Sunndalsøra for later infection with *A. salmonicida*. All 4800 individuals were marked by a combination of fin clipping and cold branding specific to each family (Gunnes & Refstie 1980) and transported to VESO Vikan AkvaVet, Namsos, Norway (ca. 400 km from Sunndalsøra). Here, the 4800 individually marked juveniles were kept together in a single 6 m³ tank with a fresh water supply at 12 °C.

Infection of the test fishes with *A. salmonicida* was performed by means of cohabitation within the test tank. Fishes ($n=300$) of the same origin as the test fishes were inoculated by intraperitoneal injection of approximately 50 000 viable cells of Gram-negative proteobacteria *A. salmonicida* subsp. *salmonicida* (Gjedrem *et al.* 1991). The 300 infected fishes (cohabitants) were released into the tank with the 4800 test fishes on 3 January 1996. The test lasted for approximately three weeks. Mortality was recorded daily and all dead fishes were deep frozen immediately. Out of the fishes that had died during the furunculosis outbreak, 10% were examined bacteriologically and *A. salmonicida* subsp. *salmonicida* was isolated in all cases.

To examine whether MHC genotypes are associated with resistance or susceptibility to furunculosis, we sampled families with high resistance (HR, < 35% mortality) and low resistance (LR, > 80% mortality) to the disease from the challenge tests. Both individuals that had died and those that had survived the infection were sampled from HR and LR families (table 1). In addition, we also sampled uninfected juveniles from HR and LR families kept at Sunndalsøra. These sampled juveniles had not been exposed to the bacterium, although their full-sibs had taken part in the challenge test at Vikan. The generation time of these farmed salmon is four years, hence the parents of the tested juveniles were hatched in 1991. These parental fishes had not themselves been exposed to *A. salmonicida* and they were not selected for increased disease resistance.

(b) Polymerase chain reaction amplifications

DNA was phenol–chloroform extracted from homogenized liver or muscle tissue. For DGGE, exon 2 was amplified using the primers TVS 4501 and GC-clamped MG 14 (Langefors *et al.* 2000) in a Perkin-Elmer-Cetus 9600 (Perkin Elmer, Brauchberg, NJ, USA). The 25 µl polymerase chain reaction (PCR) mixture contained 100 ng of DNA template, 20 pmol of each primer, 0.9 µg bovine serum albumin, 1 × PCR reaction buffer, 25 nmol MgCl₂, 125 µM dNTP and 0.2 units Taq polymerase (Perkin Elmer). The conditions for the PCR were as follows: denaturation for 2 min at 94 °C followed by 30 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 56 °C and primer extension for 30 s at 72 °C. The final extension was for 10 min at 72 °C. The PCR products showed one distinct band with an approximate length of 300 bp on a 2% agarose gel.

(c) Denaturing gradient gel electrophoresis

MHC class IIB genotypes were determined through DGGE screening. DGGE was performed using 5% 19:1 acrylamide–bisacrylamide parallel gels containing 1 × TAE buffer and a gradient of urea and formamide (Myers *et al.* 1987). A gravitational gradient maker was used to cast the gels. Electrophoresis was performed in 1 × TAE buffer heated to 60 °C, in electrophoresis cells and a buffer tank (C.B.C. Scientific Company, Inc., Delmar, CA, USA). For each sample, 10–15 µl of PCR products were run on gels overnight for 15 h at 90 V. All individuals were genotyped with a urea gradient of 20–45%. Three different alleles gave identical DGGE bands, and individuals carrying these bands were also examined on a gel with a 30–60% gradient, where the three alleles separated into three different DGGE bands. At the latter gradient, however, the resolution of the other fragments was inferior. Gels were stained with ethidium bromide (0.2 mg ml⁻¹ 1 × TAE) for

15 min and the DNA was visualized in a FluorImage SI (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

(d) Sequencing

Exon 2 sequences were distinguished for at least two copies of each DGGE band from unrelated individuals by PCR amplification of excised DGGE bands. Desired DGGE fragments (two, or occasionally one, from each of 25 individuals selected for characterization of all alleles) were identified, cut from the gel, mixed with 160 µl double-distilled water (ddH₂O), extracted by keeping the samples at -80 °C for 1 h, 25 °C for 1 h, -80 °C for 1 h and 4 °C for 2–3 h, and used as a template in a PCR with the primers TVS 4501 and ÅL 1002 (Langefors *et al.* 2000). The PCR conditions were identical to those for amplification of exon 2 for DGGE as described in §2(c), except that the annealing temperature was 53 °C. The PCR products were ethanol precipitated, dissolved in 12–16 µl ddH₂O and used as templates for direct sequencing in the ABI PRISM 310 (Perkin Elmer Corporation, CA, USA). For each DGGE fragment, we sequenced in both directions using TVS 4501 and ÅL 1002 as primers for the 5' and the 3' strands, respectively. Sequencing reactions were run in an ABI PRISM 310 genetic analyser and edited with the gene scan analysis software (Perkin Elmer).

(e) Statistical tests

Heterozygosity levels were compared both between HR and LR broods and between surviving, dead and uninfected individuals. Broods were classified as heterozygous if all sampled individuals were heterozygous while broods containing at least one homozygous individual were classified as homozygous. Tests for genetic differences between samples (F_{ST}) were made with the program GENEPOP, which gives an unbiased estimate of the p -value for population differentiation using a Markov-chain method (Raymond & Rousset 1995). This method is especially suited to unbalanced samples and cases with many alleles per loci and small sample sizes. The Markov-chain analyses were set to 1000 dememorizations, 75 batches and 1000 iterations per batch. Differences in the frequencies of each allele were tested using Fisher's exact test with and without sequential Bonferroni corrected significance levels (Rice 1989) using each individual ($n=120$) and each brood ($n=22$) as independent variables. Odds-ratio tests were performed in SYSTAT 8.0 (Wilkinson 1996) and confidence intervals were calculated manually (Sokal & Rohlf 1998).

(f) Sequence analysis

Sequences were aligned manually in the Genetic Data Environment software. Genetic distances were calculated using the Jukes & Cantor (1969) method in the software program MEGA (Kumar *et al.* 1993). The number of non-synonymous substitutions per non-synonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) were calculated using the method of Nei & Gojobori (1986) and tested against the t -distribution with infinite degrees of freedom (Kumar *et al.* 1993). The putative amino-acid residues involved in antigen binding in Atlantic salmon were based on the corresponding antigen-binding sites identified in humans (Brown *et al.* 1993).

3. RESULTS

Nine different MHC class II exon 2 alleles were identified from the 120 analysed individuals. Among the 271 nucleotides, 38 (14%) nucleotide positions were variable

(figure 1). At each variable codon, up to three different amino acids were observed. All nine alleles encoded distinct amino-acid sequences. Out of the total 90 positions, 24 (27%) amino acids were variable, in comparison with 16 (64%) out of the 25 positions of the antigen-binding sites (figure 2).

The number of non-synonymous substitutions per non-synonymous site, d_N , was 4.4 times higher than the number of synonymous substitutions per synonymous site, d_S , in the antigen-binding site ($t=2.89$, $p < 0.01$). The corresponding ratio for the non-antigen-binding-sites was 1.2 ($t=0.29$, $p > 0.05$).

(a) High- versus low-resistance families

Out of all the families, 66.7% were heterozygous (i.e. all examined individuals in a brood were heterozygous) for exon 2 of the MHC class IIB. In all, 77% of LR families were heterozygous, compared with 55% of HR families (Fisher's exact test, $p > 0.05$). MHC class IIB allele frequencies differed significantly between HR and LR families (figure 3, $F_{ST}=0.005$, $p=0.044$). The e allele was significantly more frequent in individuals from HR families than in individuals from LR families (Fisher's exact test, $p < 0.001$, significant after Bonferroni corrections, $n=120$) and it occurred more frequently in HR families than in LR families (Fisher's exact test, $p=0.063$, $n=22$ broods). The odds-ratios of allele e were 0.20 for individuals (99.9% confidence intervals 0.05–0.73) and 0.083 for broods (95% confidence intervals 0.008–0.89). A brood with the e allele had a 12-fold (1/0.083) higher chance of becoming a HR brood than a brood lacking the e allele ($p < 0.05$). Although the frequencies of the j allele did not differ significantly between HR and LR families (Fisher's exact test, $p > 0.05$), the odds-ratio for the j allele was significant at the individual level (2.30 for individuals, 95% confidence intervals 1.001–5.28). The odds-ratio for the j allele between broods was 3.06 (95% confidence intervals 0.57–16.61, not significant).

(b) Comparisons of dead, surviving and uninfected fishes

Heterozygosity levels did not differ between dead fishes (94%) and fishes surviving the challenge test (100%) (Fisher's exact test, $p > 0.05$). Heterozygosity in the uninfected fishes examined was 80%.

Frequencies of both the i and the j alleles differed significantly between dead, surviving and uninfected fishes (Fisher's exact tests, i , $p < 0.001$, significant after sequential Bonferroni corrections; j , $p=0.041$, not significant after sequential Bonferroni corrections; table 2).

(c) Sequence analysis

Construction of a phylogenetic tree, based on the neighbour-joining method, showed that allele c has the shortest distance to allele e , allele b has the shortest distance to allele i and alleles c and e have the shortest distance to allele j (tree not shown). Hence, pairwise comparisons were performed between the sequences of these pairs of alleles. Allele c and allele e differ at a single nucleotide, resulting in a glutamine–histidine substitution positioned between two residues included in the antigen-binding site (figures 1 and 2). Allele j differed from alleles

	1	11	21	31	41	50
a	GATGGATATT	TTTATCAGGT	TGTGAGACAG	TGCCGATACT	CCTCAAAGGA	
bG.A.....	
cG.A.....	
dTA.	GA.....	
eG.A.T..	
fAG	G...TC.G..	
gTA.	GA...C...	
iT.....	
jG.A.....	
	51	61	71	81	91	100
a	CCTGCAGGGT	ATAGAGTTTA	TAGACTCTTA	TGTTTTCAAT	AAGGCTGAAT	
b	
c	
dG.	.TAC...G..	C.....	
e	
f	
gG.	.TAC...G..	C.....A	
iC.....	C.....A	
j	
	101	111	121	131	141	150
a	ATGTCAGATT	CAACAGCACT	GTGGGGAAGT	ATGTTGGATA	CACTGAGTAT	
bCTG	
cCTG	
d	.A.....	
eCTG	
f	
g	.A.....	T.....C..	
i	.A.....CTG	
j	T.....CTG	
	151	161	171	181	191	200
a	GGAGTGAAGA	ATGCAGAAGC	CTGGAACAAA	GGTCCTGAGC	TGGCTGGAGA	
bT..	
cT..	
d	
eT..	
f	
g	
i	
jT..	
	201	211	221	231	241	250
a	GCTAGGGGAG	CTGGAGCGTT	ACTGTAAGCA	TAACGCTCCT	ATCTACTACA	
b	T.....GA.	C..C.....C	
c	T.....G..	
dG	T.....G.....	
e	T.....G..	
fG	T.....G.....	
gT.TTG.....	
i	T.....T.GA.	C..C.....	
jTG.....	
	251	261	271			
a	GCGCCATACT	GGACAAGACA	G			
b				
c	.T.....				
d				
e	.T.....				
fC....				
g				
i	.A.....				
j				

Figure 1. Exon 2 sequences of the MHC class IIB alleles of Atlantic salmon. Dots indicate identity with the top sequence.

c and *e* at six and seven nucleotides, respectively, spread over the entire exon. Four of the six amino-acid substitutions were located among the ABS.

4. DISCUSSION

This is, to our knowledge, the first study on MHC variation and disease resistance in a lower vertebrate, and among the first finding a correlation between MHC class II allelic variation and resistance to a bacterial pathogen. The results presented strongly indicate that MHC class IIB alleles are of importance for individual fitness in Atlantic salmon. The distributions of MHC class IIB alleles were significantly different between

families with high resistance and low resistance to infection with *A. salmonicida*. Allele *e* was significantly more prevalent among HR families than among LR families and a brood carrying the *e* allele had a 12-fold higher probability of becoming an HR family than a brood lacking the *e* allele. In addition, this specific allele was more prevalent among uninfected and infected surviving individuals than among infected dead individuals, although the difference was not significant. Allele *i* was absent among dead individuals but occurred in 11% of infected surviving and uninfected fishes (table 2). Allele *j* was more common among LR families and in infected dead individuals than among HR families and in uninfected and infected surviving individuals, but these

	1	11	21	31	41	50
		* * *		* * *	**	*
a	DGYFYQVVRQ	CRYSSKDLQG	IEFIDSYVFN	KAEYVRFNST	VGKYVGYTEY	
bE.....L	
cE.....L	
dHMM.....L.T.....	Q.....I.....	
eEH.....L	
fR.SE.....	
gHMMT.....L.T.....	Q.....NI.....F.....H	
iF.....H.....	Q.....NI.....F.....L	
jE.....F.....L	
	51	61	71	81		
	*	**	* * * * *	* * *	** * **	
a	GVKNAEAWNK	GPELAGELGE	LERYCKHNAP	IYYSAILDKT		
bV.....F.....D	LH.R.....		
cV.....F.....A		
dV.....D.....		
eV.....F.....A		
fV.....D.....L.....		
gV.....F.....D.....	
iF.....VD.....LH.....T.....	
jV.....L.....D.....		

Figure 2. Putative amino-acid sequences for MHC class IIB exon 2 alleles of Atlantic salmon. Dots indicate identity with the top sequence. Codons involved in antigen binding in humans (Brown *et al.* 1993) are denoted by asterisks.

Table 2. Percentages of the MHC class II alleles *e*, *i* and *j* in juvenile Atlantic salmon that were infected and had died or survived infection with *Aeromonas salmonicida* or that were uninfected

(Results of Fisher's exact tests are given before (*p*) and after (Bonferroni) sequential Bonferroni corrections (Rice 1989).)

	<i>e</i>	<i>i</i>	<i>j</i>
dead	23	0	19
surviving	28	11	9
uninfected	36	11	8
<i>p</i>	> 0.05	< 0.001	0.041
Bonferroni	not significant	significant	not significant

differences were only significant before Bonferroni corrections for multiple tests.

The multiple alleles commonly found at the MHC locus result in low allele frequencies and relatively low sample sizes, making it statistically problematic to find associations between MHC alleles and resistance to an infectious disease (Hill 1998). The lower number of alleles in MHC class II than in MHC class I may explain why associations have been found between susceptibility to infectious diseases and MHC class II rather than MHC class I alleles (Hill 1999). Given the small sample sizes for many of the nine alleles, it is remarkable that a significant association was found between the *e* and *i* alleles and resistance to furunculosis, as well as a tendency to an association between the *j* allele and susceptibility to the disease. This provides strong evidence of an actual association between certain MHC alleles and resistance to furunculosis in Atlantic salmon.

Association between a gene and a disease can be due to effects of the gene itself or can arise if the studied gene is in linkage disequilibrium with another gene that causes the resistance. We cannot exclude the possibility that another linked gene has caused the observed association. Our study was performed in a confined population with an unknown degree of inbreeding. The conclusion that

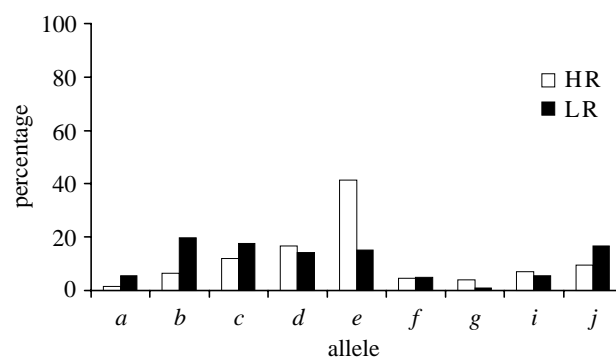


Figure 3. Distribution of MHC class IIB alleles in high-resistance families (open bars) and low-resistance families (filled bars) of Atlantic salmon; $F_{ST} = 0.005$, $p = 0.044$.

allelic variation at the MHC class IIB is correlated with resistance to furunculosis must therefore include any co-segregating variable genes.

The observed associations between the *e*, *i* and *j* alleles and resistance/susceptibility to furunculosis support the hypothesis that frequency-dependent selection is important for the maintenance of MHC variation (Slade & McCallum 1992). In addition, heterozygous individuals do not have increased resistance to furunculosis, hence this study does not support heterozygous advantage as the main mechanism for maintaining MHC variation. Associations between MHC heterozygosity and infectious diseases have been found in only two studies (Thursz *et al.* 1997; Carrington *et al.* 1999), while a larger number of studies report associations between MHC alleles and disease resistance, which also indicates that frequency-dependent selection is more important than heterozygous advantage (Hill *et al.* 1991; Paterson *et al.* 1998; Jeffery *et al.* 1999; Hill 1999). However, most studies deal with single viral, bacterial or parasitic agents (Hill *et al.* 1991; Thursz *et al.* 1997; Jeffery *et al.* 1999; Carrington *et al.* 1999) and it is possible that studies combining two or more pathogens would be able to find evidence for heterozygous advantage. Other viral and bacterial pathogens are likely to be

associated with other MHC alleles. There are a large number of different strains within the species *A. salmonicida* (Umelo & Trust 1998) and future studies will show whether the *e* allele, identified here, is also associated with resistance to other *A. salmonicida* strains.

The single nucleotide substitution in alleles *c* and *e*, replacing glutamine with the basic histidine, may have an important effect on peptide-binding efficiency. Since allele *e* has a unique motif at positions 5 and 6 (amino acids EH, figure 2) while allele *c* shares the motif EQ with alleles *b* and *j* at these positions (figure 2), it seems likely that a single-point mutation in allele *c* gave rise to allele *e* (figure 1). It is often assumed that new alleles are lost due to random genetic drift. However, under the theory of negative frequency-dependent selection, rare alleles, both new and old, have a selective advantage and will increase in frequency (May & Anderson 1990). Allele *e* could be an example of a 'new' allele that has arisen and been maintained through frequency-dependent selection due to its association with resistance to furunculosis.

In the present study, we have found associations between certain MHC class II alleles and the bacterial infection, furunculosis. These results suggest that directional or, eventually, frequency-dependent selection is acting on the MHC. Association between a gene and a disease can be due to effects of the gene itself or can arise if the studied gene is in linkage disequilibrium with another gene that has causative effects. We cannot exclude the possibility that another linked gene has caused the observed association. However, the peptide-presenting function of the MHC class II molecule indicates that it is the MHC itself that causes the association.

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