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Interactions of Yersinia enterocolitica biotype 4 and 1A strains with macrophages in vitro

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Summary

The interaction of Y. enterocolitica strains belonging to 4 and 1A biotypes with RAW264.7 murine macrophage-like cell monolayers was studied. Y. enterocolitica strains from humans and pigs were characterized in terms of their internalization and survival within RAW264.7 cell monolayers and their ability to escape from the cells. Y. enterocolitica biotype 4 strains invaded macrophage cell monolayers to a significantly higher degree than biotype 1A strains. However, biotype 1A strains exhibited a greater level of survival in macrophages than biotype 4 strains. All Y. enterocolitica strains tested demonstrated the ability to escape from macrophages. The mechanisms that allow Y. enterocolitica biotype 1A to survive within macrophages may contribute to the virulence of these organisms.

Keywords: Yersinia enterocolitica

Yersinia enterocolitica is an important human pathogen which causes a variety of disorders from nonspecific diarrhoea to invasive diseases such as terminal ileitis, mesenteric lymphadenitis, and septicemia (1). Serotypes, biotypes and phage types of *Y. enterocolitica* isolated from pigs have been reported to be the same as those isolated from humans. Therefore, pigs are recognized as a primary source of human infection (7, 13-15). *Y. enterocolitica* biotype 4 strains, pathogenic for people, are isolated in Europe most frequently. Biotype 4 is the most frequently isolated biotype from patients in Poland as well (18), whereas biotype 1A is often isolated from livestock (pigs, cattle, sheeps). Recently these strains have also been isolated from humans with yersiniosis (14, 15, 25). The virulent *Yersinia* strains carry an approximately 70 kb plasmid, termed pYV, which codes for YadA and Yersinia outer (Yop) proteins. YadA promotes adhesion to eukaryotic cells (5). Yops proteins interfere in cellular signalling pathways that lead to innate and adaptive immune response (4). Apart from the proteins encoded on the plasmid, the essential virulence factors of the pathogenic strains are also encoded on a chromosome. Among them, the most important in *Y. enterocolitica* pathogenesis are those encoded by ail, yst, and myfA genes. The ail gene product, called Ail (Attachment invasion locus), is a small outer membrane protein. Ail promotes bacterial adhesion to and invasion of cultured epithelial cells (19). The heat-stable enterotoxin Yst, a product of *yst* gene, damages intestinal epithelium (19). The myfA gene encodes a major subunit of antigen Myf, which forms a fibrillar structure. Myf has been found to promote the colonization of the intestine and allows the secretion of Yst enterotoxin (22).

These *Y. enterocolitica* virulence genes, localized on both pYV plasmid and chromosome, are the markers often used in diagnostic molecular techniques, i.e. PCR, and multiplex-PCR (6, 23). Biotype 1A strains of *Y. enterocolitica* are generally considered to be avirulent because they are deficient in the genotypic and phenotypic markers associated with virulence of highly pathogenic strains of *Y. enterocolitica* (22), although in some of these strains the presence of chromosomal genes ystB and myfA was shown. These genes may contribute to virulence of *Y. enterocolitica* biotype 1A strains (8, 17, 22). The clinical manifestations of infection with biotype 1A strains do not differ from the clinical symptoms elicited by the strains of other pathogenic biotypes. The mechanisms of pathogenicity of biotype 1A strains are poorly understood (22).

In previous studies (13, 14, 25) based on the presence or absence of the yadA, ail, ystA, ystB, and myfA genes, it was identified six main genotypes among 130 human and swine *Y. enterocolitica* strains. These

strains represented biotypes 4 and 1A. The aim of the present study was to investigate the interaction of these *Y. enterocolitica* strains with macrophage cells and to compare their internalization and survival efficacy within macrophages. The ability of these strains to escape from the macrophage cells was also evaluated. These bacterial activities have been previously shown by others authors to be strictly correlated with the virulence of Y. *enterocolitica* (3, 9, 10, 22).

Materials and methods

Bacterial strains and growth conditions. The bacterial strains, used in this study, are characterized in tab. 1. The strains were isolated from the faeces of patients with intestinal yersiniosis at the Department of Microbiology and Immunology, Children's Health Centre in Warsaw, and from swine tonsils and throat swabs at the Veterinary Hygiene Research Station in Łomża. All tested strains of *Y. enterocolitica* were biotyped according to the revised scheme of Wauters et al. (24). For the experiments, *Y. enterocolitica* strains were grown in LB medium with shaking at 25°C overnight. Then, 200 µl of the bacterial culture was inoculated to 9.8 ml of the fresh LB medium supplemented with 20 mM $MgCl_2$ and 20 mM sodium oxalate and incubated with shaking for 2 h at 25°C, followed by further incubation at 37°C for 2 h to induce the expression of Yops (3). After incubation, the organisms were harvested by centrifugation at 12,000 g for 2 min., washed with phosphate-buffered saline (PBS), and resuspended in PBS to a concentration of 10^8 cfu/ml.

Macrophages cell culture and conditions of infection. The RAW264.7 murine macrophage-like cell line was maintained at 37°C in 5% CO_2 atmosphere in a Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and antibiotic-antimycotic solution (100 units penicillin, 100 µg streptomycin, 0,25 µg amphotericin B per 1 ml of the medium), and supplemented with 10% (v/v) heat-inactivated fetal bovine serum. All cell culture media and components were purchased from Sigma-Aldrich. Macrophages cell culture between passage 10 to 15 was used in the experiments. Twenty-four hours before infection of macrophages, the cells were seeded at approximately 1×10^4 cells/well in a 96-well tissue culture plate (Nunclon Micro-Well Plates, Biokom System) and cultured to semi-confluency (2×10^4 cells/well) at 37°C in an atmosphere of 5% CO_2. Just before infection with *Y. enterocolitica*, the tissue culture medium was removed and cells were washed with PBS without Ca^{2+} and Mg^{2+} (Biomed). The bacteria were diluted 1 : 100 in an infection medium (DMEM without antibiotics and antimycotic) and 100 µl of the bacterial suspension was added to each well at a multiplicity of infection (MOI) of 50 bacteria per eukaryotic cell, and incubated for 1 h at 37°C in a 5% CO_2 atmosphere.

Tab. 1. Characteristics of *Y. enterocolitica* strains used in this study

Strain	Source	Biotype	Presence of virulence genes				
			yadA	ail	ystA	ystB	myfA
1	Faeces of humans	4	+	+	+	–	+
2		4	+	+	+	–	+
3		4	+	+	+	–	+
4		4	+	+	+	–	+
5		4	–	+	+	–	+
6		4	–	+	+	–	+
7		4	–	+	+	–	–
8		1A	–	–	–	+	+
9	Tonsils, throat swabs of pigs	4	–	–	+	–	+
10		1A	–	–	–	+	–
11		1A	–	–	–	+	–
12		1A	–	–	–	+	+
13		1A	–	–	–	+	+

Investigation of *Y. enterocolitica* invasion and survival within macrophages. After 1 h of infection, the cells were washed with PBS to remove non-adherent bacteria, and 100 µl of fresh tissue culture medium containing gentamicin at a concentration of 100 µg per ml was added to each well for 1 h to kill extracellular bacteria. The macrophages were then washed twice with PBS and lysed for 30 min. with 150 µl of 0.05% trypsin in PBS with 1% Tween-20 per well to release the intracellular bacteria. Then, 50 µl of this suspension was serially diluted in PBS, and spread on LB agar plates. The number of viable bacterial cells was determined after growth at 25°C for 48 h. The number of the bacteria, which invaded RAW264.7 macrophages was designated as the initial count (T = 0 h). In the parallel microplate wells, the medium containing 100 µg gentamicin per ml was replaced with the medium containing 20 µg of gentamicin per ml, and the infected cell monolayers were incubated for a further 24 h. The macrophages were then washed with PBS and lysed for 30 min. with 150 µl of of 0.05% trypsin in PBS with 1% Tween-20 per well to release the intracellular bacteria. The number of bacteria was then enumerated by plating as described above. The number of the intracellular bacteria was considered to be the final count (T = 24 h). The percentage of the surviving bacteria was calculated from the number of intracellular bacteria after 24 h of infection (the final count) divided by the number of intracellular bacteria after 1 h of infection (the initial count) times 100.

Assessment of the bacterial escape from macrophages. After 1 h of infection, the RAW 264.7 macrophages were washed with PBS, and a fresh cell culture medium – DMEM containing 100 µg gentamicin per ml was added for 2 h to kill extracellular bacteria. Gentamicin was removed by two washes with PBS, and the cells were incubated in a fresh cell culture medium. The ability of bacteria to escape from cells was established 18 h after the removal of gentamicin by plating the culture medium and enumerating the bacteria (9).

Statistical analysis. Data from at least two independent experiments, each run in triplicate, were analysed by least-squares analysis of variance using the GLM procedure of SAS (20). Multiple comparisons of the group means and identification of differences were performed using Duncan's multiple-range test and SAS software. A critical value of $p \leq 0.05$ was used for all analyses.

Results and discussion

RAW264.7 macrophage-like cell monolayers were coincubated with 13 strains of *Y. enterocolitica*. The strains represented six genotypes with respect to their virulence determinants. The percentage of *Y. enterocolitica* internalized cells was determined after 1 h infection in relation to the initial number of bacteria added to each well. The seven strains (No. 1-6, and 9) inva-

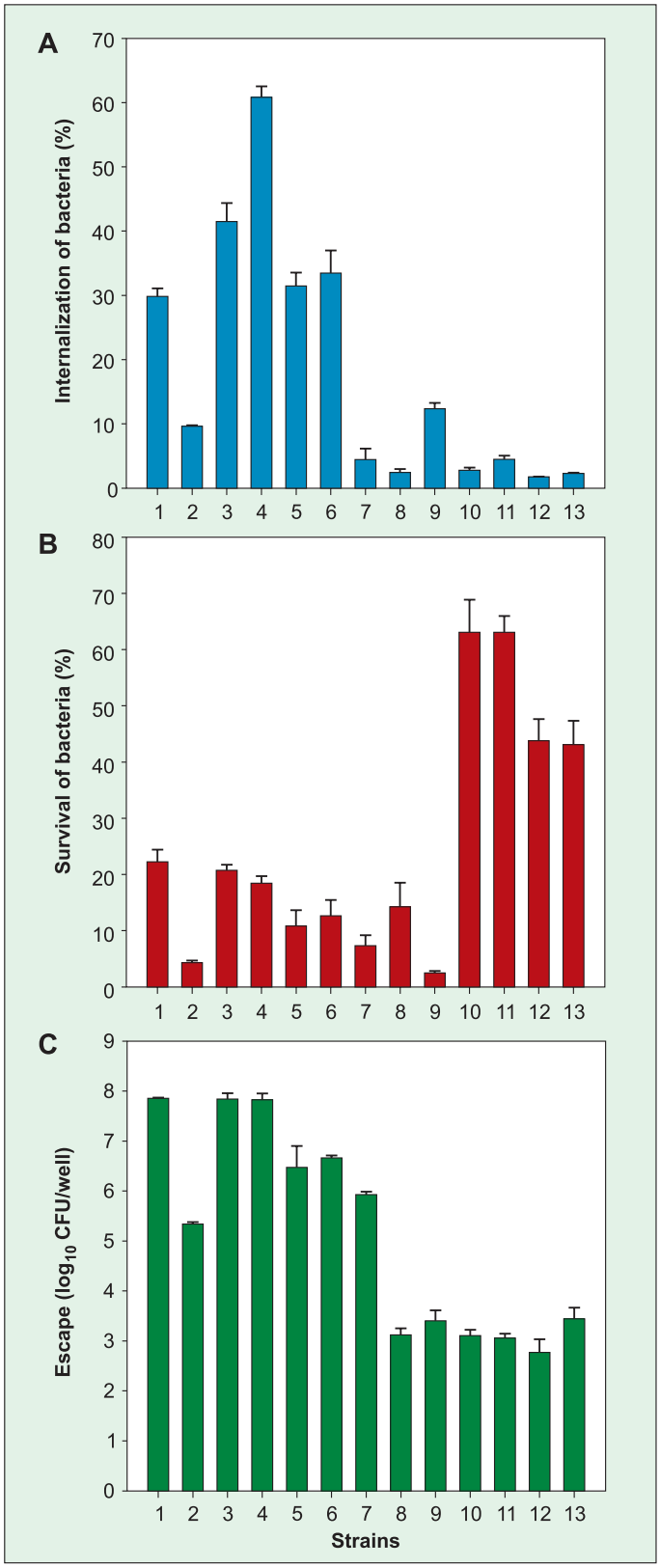

Fig. 1. Interaction of *Y. enterocolitica* strains with RAW264.7 macrophages

Explanations: (A) Percentages of the bacteria internalized by macrophages after 1 h of infection relative to the original inoculum. (B) Survival of the strains within macrophages. Results are expressed as the percentage of the number of bacteria recovered from the cell monolayers after 24 h relative to the number of the bacteria recovered after 1 h of infection. (C) Escape of bacteria from the cell monolayers at 18 h post-infection. Experiments were performed at least two times, each in three repetitions

ded macrophages in significantly higher percentages than the remaining strains (fig. 1A). All these strains represented biotype 4 and three genotypes: $yadA^+$ ail^+ $ystA^+$ $myfA^+$, $yadA^-$ ail^+ $ystA^+$ $myfA^+$, and $yadA^-$ ail^- $ystA^+$ $myfA^+$.

To determine the ability of *Y. enterocolitica* strains to persist in macrophages, the infected eukaryotic cells were lysed after 24 h of incubation in DMEM containing gentamicin at a concentration of 20 µg/ml, and the intracellular bacteria were counted. For the strains tested, significant differences were observed in the number of the viable intracellular bacteria (fig. 1B). Four of the five strains, which invaded macrophages in the lowest number showed significantly higher survival in macrophages than the other strains (except strain No. 8). All these strains belonged to biotype 1A and represented two genotypes: $ystB^+$ $myfA^-$, and $ystB^+$ $myfA^+$.

All *Y. enterocolitica* strains tested showed the ability to escape from macrophages (fig. 1C). For the strains, which invaded macrophages with the highest efficiency and demonstrated poor ability to survive in eukaryotic cell monolayers, the high numbers of extracellular bacteria were observed at 18 h post-infection.

The strains of biotype 1A and biotype 4 were compared in terms of their ability to invade macrophages, their survival within and their escape from the eukaryotic cells (fig. 2). The average percentage of the internalized cells from strains of biotype 4 was significantly higher than the average of the internalized cells from strains of biotype 1A. The percentage of the bacteria recovered from macrophages ranged from 4.6 to 61% of the orginal inoculum for biotype 4 strains and from 1.9 to 4.7% for biotype 1A strains (fig. 2A). The biotype 1A organisms, on the contrary, survive within macrophages in significantly higher numbers than biotype 4. The percentage of the bacteria recovered from macrophages ranged from 14.5 to 78% for biotype 1A strains and from 2.7 to 22.5% for biotype 4 strains (fig. 2B). The biotype 4 organisms escaped from macrophages and grew in the cell culture medium outside the cells in significantly higher numbers than biotype 1A organisms. The number of the extracellular bacteria ranged from 3.4 to 7.9 $\log_{10}$ for biotype 4 strains, and from 2.8 to 3.3 $\log_{10}$ for biotype 1A strains (fig. 2C). The correlation coefficient for the average number of bacteria from the particular *Y. enterocolitica* strains that escape from the cell monolayers versus the average percentage of the bacteria of the corresponding strains that were internalized within macrophages was high and equal to 0.89.

In this study, four genotypes with respect of yadA, ail and myfA genes were investigated within *Y. enterocolitica* strains representing biotype 4. The strains of various genotypes differed from each other in the ability to invade, survive within and escape from macrophages. A direct link between the genotype of the strain tested and its putative pathogenicity can not be drawn based solely on its interaction with macrophages in cell

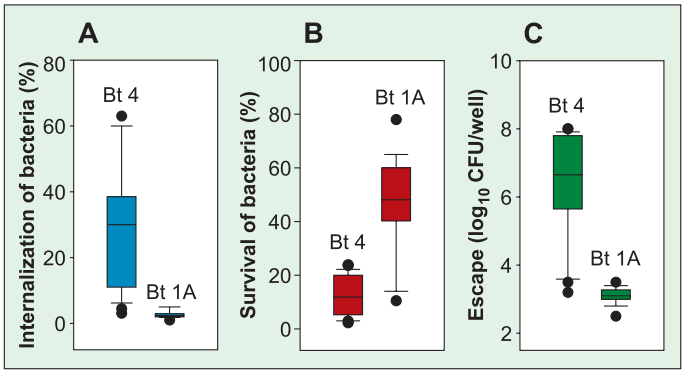

Fig. 2. Comparison of the abilities of *Y. enterocolitica* strains from biotype 1A (Bt 1A) and biotype 4 (Bt 4) to invade (A), survive within (B) and escape (C) from RAW 264.7 macrophage cell line

Explanations: The data from two independent experiments, each performed in triplicate, are presented as box plots showing the median, which is marked as the line across each box. The lower and the upper boundaries of each box represent the 25th and 75th percentiles, respectively. The whiskers below the boxes mark the 10th percentile, the whiskers above the boxes mark 90th percentile. The black dots indicate values outside this range. In all plots, the differences between biotype 4 and biotype 1A are significant ($p \leq 0.05$ by Duncan's multiple range test)

culture, however statistically significant differences in invasion, survival and escape of *Y. enterocolitica* strains of various genetic background could be observed between groups of strains harbouring the particular virulence gene when compared to the strains, which lacked the gene (tab. 2). *Y. enterocolitica* strains, which possessed sequences homologous to yadA and myfA genes were internalized more efficiently than those, which lacked these sequences. The strain which lacked the ail gene was internalized in lower number when compared to the strains which possessed this gene but this difference was not statistically significant. *Y. enterocolitica* strains of biotype 4 which possessed the yadA and ail genes survived in significantly higher numbers than those, which lacked these genes. No significant differences were observed between the average number of the cells of the strains with or without myfA gene that survived within and escaped from the cell monolayers. The organisms, which possessed other virulence-asociated genes (yadA and ail) escaped from macrophages in significantly higher numbers than the organisms deficient in these genes.

Tab. 2. Internalization, survival within and escape from RAW254.7 macrophages of *Y. enterocolitica* strains with different virulence genes

Virulence gene	Internalized bacteria (%)*	Bacterial survival (%)**	Bacterial escape (log_{10}cfu/well)***
yadA⁻	20.6[a]	8.2[a]	5.6[a]
yadA⁺	35.6[b]	16.7[b]	7.2[b]
ail⁻	12.5[a]	2.7[a]	3.4[a]
ail⁺	30.4[a]	13.8[b]	6.9[b]
myfA⁻	4.6[a]	7.6[a]	6.0[a]
myfA⁺	31.5[b]	13.2[a]	6.5[a]

Explanations: results are the mean of at least two experiments performed in triplicate; * – results are the percentage of the original inoculum of bacteria used to infect the cell monolayers; ** – results are the percentage of the number of organisms that invade macrophages after 1 h relative to the number of bacteria recovered from the cell monolayers after 24 h; *** – results are the number of bacteria (cfu/ml) recovered from DMEM at 18 h post-infection; means with different superscript letters differ significantly at $p \leq 0.05$

Invasion into cells, intracellular survival and ability to escape from host cells are important determinants of the virulence of pathogenic biotypes of *Y. enterocolitica* (2, 9). Previous experiments undertaken in our labs (13, 14, 25) allowed us to collect the isolates, which represent six genotypes in respect to the main *Y. enterocolitica* virulence determinants. These isolates belong to biotypes 4 and 1A. In this study, 13 isolates of six genotypes were investigated with regard to their internalization and survival within macrophages and their ability to escape from these cells. Among eight isolates of biotype 4, four carry pYV, and four possess the sequences homologous to ystA and ail or myfA genes. The isolates of biotype 1A used in the experiments carry ystB gene and three possess the sequence homologous to myfA gene in their chromosomes. All *Y. enterocolitica* strains tested invade macrophages, although to varying extent. The highest number of bacteria internalized has been found in isolates that carry yadA, ail, ystA, myfA genes. Grosdent and coworkers (10) have previously shown that YadA, *Y. enterocolitica* adhesin, support the entry of the organisms into macrophages in the absence of opsonins.

In this study, a multiparametric analysis revealed that the presence of the sequences that were homologous to at least one of the following genes yadA or myfA in the genomes of the biotype 4 strains tested, influenced the percentage of the internalized bacteria inside macrophages – these strains invade the cell monolayers more efficiently than those which lacked the sequences. YadA is an adhesin, which mediates specific binding of *Y. enterocolitica* to collagen, laminin and cellular fibronectin. This adhesin mediates indirect binding of yersiniae to β1 integrin through fibronectin-β1 integrin bridging. Interaction of YadA with β1 integrins activates focal adhesion kinase, leading to tyrosine phosphorylation of other components of the focal adhesion complex (11). MyfA, a major structural subunit of Myf, is 44% identical at the DNA level to the pH6 antigen of *Y. pestis* and PsaA of *Y. pseudotuberculosis*, which also has a fibrillar structure and mediates thermoinducible binding of *Y. pseudotuberculosis* to tissue culture cells (19). Myf also promotes the colonization of the intestine and allows the activity of enterotoxin Yst (12). Ail has been shown to confer an attachment and invasion phenotype to *Escherichia coli* K-12, and this suggests that *ail* may be an important factor during *Y. enterocolitica* invasion process (19). However, in our experiments, we did not

observe that strains, which possess the ail gene, invaded RAW264.7 murine macrophages more efficiently when compared to the strains that lacked this gene.

Several studies have demonstrated that *Y. enterocolitica* can persist within professional phagocytes (3, 16, 21). However, the bacteria of the strains bearing yadA, myfA genes, which invaded macrophages to a greater extent, escaped efficiently from the cell monolayer. On the contrary, the bacteria from biotype 1A strains, isolated from pigs that invade macrophages poorly survived within the cell monolayer in significantly higher numbers than did biotype 4 strains. Biotype 1A isolates, which are able to persist within epithelial cells and macrophages may be equipped with other, as yet unknown, mechanisms of virulence (9, 21). It has been reported that some of biotype 1A strains carry myfA gene (22). There is no available data on the interaction of these strains with macrophages, and the pathogenicity of myfA$^+$ biotype 1A isolates. In performed experiments, an analogous profile of invasion, survival and escape from macrophages has been observed for myfA$^+$ and myfA$^-$ biotype 1A strains.

In this study, among the biotype 4 isolates tested, the strains that carry yadA and ail genes displayed a significantly better ability to survive within macrophages as compared to biotype 4 strains that lacked these genes. However, the percentage of yadA$^+$ail$^+$ biotype 4 strains that survive killing by macrophages was significantly lower than those observed for biotype 1A strains. It has been suggested that survival in macrophages allows *Yersinia* to evade specific immune response and may aid in persistence in the host. Therefore, this property contributes to the virulence of these organisms (16). The findings of this study support the results of Singh and Virdi (21), which showed that *Y. enterocolitica* strains of biotype 1A are able to survive in high numbers in J774 mouse macrophages.

All the *Y. enterocolitica* strains tested, both clinical and from pigs, representing biotypes 4 and 1A had the ability to escape from macrophages. The ability of biotype 1A isolates to escape have been observed after infection of macrophages for 18 h. However, the number of viable extracellular bacteria from these strains was significantly lower than from the strains of biotype 4. This property correlated with the ability to invade macrophages, because those strains which invaded macrophages more in greater numbers escaped from macrophages in smaller numbers. The mechanism used by *Y. enterocolitica* to escape from host cells is unknown. Tennant et al. (22) have recently shown that *Y. enterocolitica* requires intact cell wall lipopolysaccharide to replicate within host cells before they can escape.

In this study, was demonstrated that even those *Y. enterocolitica* strains of biotype 1A, which lack yadA, ail, myfA genes, are able to enter, survive and multiply within RAW264.7 murine macrophages. The importance of this finding for pathogenesis of *Y. enterocolitica* strains of biotype 1A remains to be elucidated. However, the nucleic acid-based amplification techniques that rely only on yadA, ail, and myfA sequences may not detect all potentially pathogenic *Y. enterocolitica* strains. Taking into consideration the fact that mechanism that allows bacteria to survive within macrophages may be important for *Y. enterocolitica* spreading within a host, a search for genetic markers that determine these phenomena in warranted.

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