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Mycobacterium avium resists exposure to the acidic conditions of the stomach

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Abstract

Organisms of the *Mycobacterium avium* complex are common pathogens in immunosuppressed patients such as individuals with AIDS. There is evidence that in AIDS patients, the main route for *M. avium* infection is the gastrointestinal tract. The stomach is a formidable barrier to pathogens and the ability to resist exposure to pH lower than 3 has been shown to be a virulence determinant of enteric pathogens. Incubation of three clinical isolates of *M. avium* under acidic pH revealed resistance of *M. avium* grown both to the exponential and stationary phase at pH 2.2 for 2 h. Inhibition of protein synthesis had no effect on the acid tolerance. When the duration of the incubation at pH 2.2 was extended to 24 h, bacteria grown to the stationary phase had a significantly greater tolerance to acid than exponential phase bacteria. *M. avium* incubated with acid in the presence of water was significantly more resistant to pH 2.2 than *M. avium* in the presence of buffer. Pre-adaptation in water prior to exposure to acidic conditions was also associated with increased resistance to pH 2.2. Isoosmolarity of Hank's balanced salt solution appears to be responsible for the impaired resistance to acid between 2 and 24 h of incubation. These findings indicate that *M. avium* is naturally tolerant to pH < 3 and that pre-adaptation under conditions similar to the conditions where *M. avium* is found in the environment results in increased acid resistance.

Keywords: *Mycobacterium avium*; AIDS; Acid tolerance

1. Introduction

Disseminated infection with *Mycobacterium avium* occurs in patients with AIDS during the advanced stage of disease [1,2]. *M. avium* is an environmental organism, encountered in water and soil [3]. In AIDS patients, in contrast to other patient populations, the large majority of *M. avium* infection is acquired by the gastrointestinal tract [4,5], although some cases appear to be acquired through the respiratory tract [6].

To gain access to the intestinal mucosa, *M. avium* must cross the stomach barrier, one of the host's non-specific defense mechanisms. Bacteria such as *Salmonella, Shigella* and *Escherichia coli* have the ability to resist acid, requiring in some cases a pre-adaptation step to tolerate the acidic conditions [7]. Several studies have demonstrated that the above cited bacteria are able to adapt to the adverse conditions of the stomach [8,9]. For example, the ability of *Shigella* to survive low pH is dependent on the time of exposure, pH and growth phase of the bacterium [10].

A mechanism of host defense against *Mycobacterium tuberculosis* has long been assumed to be the acid pH of the stomach [11]. Gastrectomy as well as chronic gastritis, both conditions associated with decrease or absence of acid barrier, are risk factors for the development of intestinal tuberculosis [11].

On the basis of these observations and *M. avium*'s entrance through the gastrointestinal tract in AIDS patients, we sought to investigate acid resistance of *M. avium*.

2. Materials and methods

2.1. Mycobacteria

M. avium strains 101, 109 and 93344 were obtained

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from the blood of AIDS patients (101 and 109) or recovered from the liver biopsy of an AIDS patient (93344). The *M. avium* strains were grown on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI, USA) plates for 10 days at 37°C and transparent colonies were transferred to 7H9 broth supplemented with oleic acid, albumin, dextrose and catalase (OADC, Difco) and grown to either the exponential (5 days) or stationary (14 days) phase. *Mycobacterium smegmatis* mc^2155 (a gift from Dr. Williams Jacobs, Jr.) was grown to the exponential (1 day) and stationary (4 days) phase. Bacterial viability of the inoculum was determined by plating an aliquot for CFU and by performing the LIVE-DEAD assay (Molecular Probes, Portland, OR, USA) as previously described [12].

2.2. Culture conditions

Bacterial cells were harvested by centrifugation, washed once in cold Hank's balanced salt solution (HBSS) and resuspended in 7H9 broth without OADC in the presence of 0.1 N HCl solution. The pH was adjusted to either 2.2 or 6.8 and the bacterial suspension was incubated at 37°C. Viability counts were carried out at 2 h as well as 24 h as previously reported [12]. Assay conditions were based on those of the normal fasting stomach, i.e. pH lower than 3.0 and gastric emptying time of 2 h or less [13]. Previous work with other enteropathogenic bacteria had established acid tolerant strains in which $>10\%$ of the initial inoculum survived after exposure to pH 2.5 for 2 h [8]. All chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA) except where stated otherwise.

2.3. Assessing the importance of 'de novo' protein synthesis

To determine if protein synthesis is required for acid resistance, the assay for acid resistance was repeated as described [14].

2.4. Culture under different conditions

To examine whether bacteria exposed to acid either in water or HBSS (as a salt solution) resist similarly to acid, *M. avium* 101 was grown in 7H9 for 14 days (stationary phase), centrifuged and resuspended in water. Then, 0.1 ml of the final suspension was added to 0.9 ml of sterile water or HBSS. The volume was then split in two and while one half was acidified to pH 2.2, the other half did not undergo acidification. Two and 24 h after the acid was added, the number of bacteria in the suspension was quantitated. The pH of the suspensions was monitored in the beginning, 4 h after (for the 24-h time point) and at the end of the experiment and was shown to be unchanged. All the studies under different conditions were done using stationary phase bacteria.

2.5. Prior adaptation and acid resistance

In order to determine whether incubation under different conditions prior to incubation with acid would have a significant impact on acid resistance, *M. avium* strain 101 was incubated in H_2O (pH 6.8) (natural environment for the organism) or HBSS (pH 6.8) without OADC for 18 h at room temperature. HBSS contains $CaCl_2$, KCl, KH_2PO_4, $MgSO_4$, NaCl, $NaHCO_2$, Na_2HPO_4 and glucose. Then, the bacteria were centrifuged at 4°C and resuspended in 7H9 without OADC and the number of bacteria was quantitated. Following the addition of 0.1 N HCl, the pH was adjusted to 2.2 or 6.8. Bacteria were then incubated for 2 or 24 h and viability was determined.

To investigate the correlation between HBSS and H_2O and *M. avium* resistance to acid, *M. avium* was pre-incubated in H_2O, HBSS and H_2O, supplemented with NaCl (10.3 mg ml^{-1} to make water isoosmolar), 0.186 mg ml^{-1} $CaCl_2$ (concentration of Ca^{2+} in HBSS), 0.2 mg ml^{-1} MgO_4 (concentration of Mg^{2+} in HBSS), 11.5 mg ml^{-1} KCl (concentration in HBSS) or 0.1 M sucrose (pH 6.8) for 18 h and then exposed to pH 2.2 for 24 h. Control tubes without acid were maintained under the same conditions.

2.6. Statistical analysis

Results were compared at the same time points and analyzed by the Student's *t*-test or ANOVA. Statistical significance was considered when $P<0.05$.

3. Results

3.1. M. avium resistance to acid

As shown in Table 1, *M. avium* grown to the exponential phase resisted exposure to acidic pH for 2 h, without significant loss of viability. However, prolonged exposure (24 h) to pH 2.2 was associated with significant reduction of the inoculum compared to *M. avium* incubated at pH 6.8.

Table 2, however, shows that in bacteria grown to the stationary phase, even prolonged exposure to acidic conditions did not result in significant decrease in bacterial CFU. *M. smegmatis* was sensitive to pH 2.2 when cultured to the exponential phase of growth, but partially tolerant at 2 h when grown to the stationary phase.

3.2. Effect of the inhibition of protein synthesis on acid resistance

Strains 101 and 93344 were incubated in the presence of a sub-inhibitory concentration of amikacin (10 μg ml^{-1}), known to inhibit protein synthesis [14], prior to exposure to pH 2.2 for 2 h.

Table 1
Acid resistance of *M. avium* strains grown to the exponential phase of growth

M. avium strain (inoculum)	pH	Viability	
		2 h	24 h
101 ($1.0 \pm 0.1 \times 10^5$)	2.2	$1.4 \pm 0.8 \times 10^{5}$[b]	$2.6 \pm 0.5 \times 10^{4}$[a]
	6.8	$1.6 \pm 0.3 \times 10^5$	$1.8 \pm 0.3 \times 10^6$
109 ($4.0 \pm 0.7 \times 10^5$)	2.2	$3.6 \pm 0.7 \times 10^{5}$[b]	$1.6 \pm 0.2 \times 10^{4}$[a]
	6.8	$3.9 \pm 0.3 \times 10^5$	$1.3 \pm 0.3 \times 10^6$
93344 ($1.8 \pm 0.2 \times 10^5$)	2.2	$1.4 \pm 0.3 \times 10^{5}$[b]	$2.8 \pm 0.9 \times 10^4$
	6.8	$1.9 \pm 0.4 \times 10^5$	$1.0 \pm 0.3 \times 10^6$
M. smegmatis ($3.1 \pm 0.4 \times 10^5$)	2.2	$5.3 \pm 0.4 \times 10^{4}$[a]	0[a]
	6.8	$3.0 \pm 0.3 \times 10^5$	$9.9 \pm 0.4 \times 10^6$

[a] $P < 0.05$ compared with the initial inoculum.
[b] $P > 0.05$ compared with the initial inoculum.

Table 2
Acid resistance of *M. avium* strains grown to the stationary phase of growth

M. avium strain (initial inoculum)	pH	Viability	
		2 h	24 h
101 ($1.7 \pm 0.3 \times 10^5$)	2.2	$1.5 \pm 0.2 \times 10^{5}$[b]	$1.4 \pm 0.2 \times 10^{5}$[b]
	6.8	$1.8 \pm 0.2 \times 10^{5}$[b]	$3.9 \pm 0.5 \times 10^5$
109 ($1.0 \pm 0.1 \times 10^5$)	2.2	$9.6 \pm 0.2 \times 10^{4}$[b]	$8.8 \pm 0.3 \times 10^{4}$[b]
	6.8	$1.1 \pm 0.3 \times 10^5$	$2.6 \pm 0.2 \times 10^5$
93344 ($2.9 \pm 0.5 \times 10^5$)	2.2	$2.7 \pm 0.4 \times 10^{5}$[b]	$2.2 \pm 0.4 \times 10^{5}$[b]
	6.8	$2.9 \pm 0.3 \times 10^5$	$3.2 \pm 0.2 \times 10^5$
M. smegmatis ($1.4 \pm 0.3 \times 10^5$)	2.2	$8.3 \pm 0.5 \times 10^{4}$[a]	$5.5 \pm 0.3 \times 10^{2}$[a]
	6.8	$1.6 \pm 0.3 \times 10^5$	$1.9 \pm 0.2 \times 10^5$

[a] $P < 0.05$ compared with the initial inoculum.
[b] $P > 0.05$ compared with the initial inoculum.

3.3. Influence of culture conditions on acid resistance

Ingestion of contaminated water or food represents a primary route for *M. avium* infection [3]. Therefore, we examined whether ingestion with water or with salt and glucose containing fluids would have any effect on acid resistance. The results shown in Table 3 indicate that stationary phase *M. avium* when exposed to pH 2.2 in the presence of water tolerates acidity up to 24 h without significant decrease in the number of viable organisms. However, if *M. avium* is exposed to a very low pH in the presence of HBSS, viability of the inoculum decreases over time. No decrease in viability resulted from incubation of *M. avium* for 24 h at pH 6.8. Similar results were obtained with the strain 109 (data not shown).

3.4. Effect of pre-adaptation on acid resistance

In an attempt to partially mimic the real conditions that precede *M. avium* intake by the host, we suspended *M. avium* either in sterile water or buffer (HBSS) containing salts and glucose for 18 h and then exposed the two populations of organisms to pH 2.2. As shown in Table 4, viability of *M. avium* pre-incubated in water was not reduced between 2 and 24 h. In contrast, pre-incubation in HBSS resulted in a decrease of bacterial viability over 24 h. Because HBSS constitutionally differs from water by the presence of Ca^{2+} and Mg^{2+} and by its isoosmolarity, we attempted to determine which of these single elements and conditions was responsible for the decrease in resistance to acid compared to water. As shown in Table 5, incubation in H_2O made isoosmolar (with NaCl, KCl or sucrose), but not water with $CaCl_2$ or $MgSO_4$, resulted in loss of acid resistance in *M. avium*.

Table 3
M. avium resistance to acid under different conditions

M. avium 101 (initial inoculum)	pH	Viability	
		2 h	24 h
H_2O ($7 \pm 0.3 \times 10^5$)	2.2	$6.7 \pm 0.7 \times 10^5$	$6.6 \pm 0.8 \times 10^6$
	6.8	$6.8 \pm 0.9 \times 10^5$	$6.9 \pm 0.4 \times 10^6$
HBSS ($7 \pm 0.3 \times 10^5$)	2.2	$6.7 \pm 0.3 \times 10^5$	$4.7 \pm 0.9 \times 10^{4}$[a]
	6.8	$6.8 \pm 0.6 \times 10^5$	$6.4 \pm 0.4 \times 10^6$

M. avium was grown to the exponential phase in 7H9 broth for 24 h and then, 0.1 ml was resuspended in 0.9 ml of H_2O or HBSS and the suspensions were made acidic (pH 2.2). The pH of the suspension was verified in the beginning, at 4 h (for the 24-h time point) and at the end of the experiment.
[a] $P < 0.05$ for the decrease of viability compared with the initial inoculum.

4. Discussion

The ability of bacterial pathogens to withstand environmental stress, both outside and inside the host, plays a critical role in determining their success as pathogens. The role of the low pH of gastric secretions as a barrier to intestinal infections is well documented [13,15]. The ability of the bacterium to survive the acidic conditions of the stomach can contribute to virulence by increasing the likelihood of intestinal colonization. Survival in acidic pH certainly has clinical significance, because pathogens must pass through the stomach at pH < 3 for up to 2 h before colonizing the intestinal tract [13,16].

Table 4
M. avium resistance to acid following pre-adaptation in water or buffer

M. avium 101 (initial inoculum)	pH	Viability	
		2 h	24 h
H_2O ($2.0 \pm 0.2 \times 10^5$)	2.2	$2.3 \pm 0.5 \times 10^5$	$1.9 \pm 0.2 \times 10^5$
	6.8	$2.4 \pm 0.3 \times 10^5$	$2.1 \pm 0.4 \times 10^5$
HBSS ($2.8 \pm 0.3 \times 10^5$)	2.2	$2.4 \pm 0.2 \times 10^5$	$8.1 \pm 0.5 \times 10^{4}$[a]
	6.8	$2.6 \pm 0.3 \times 10^5$	$2.4 \pm 0.4 \times 10^5$

Bacteria were grown in 7H9 broth and then harvested and pre-adapted in H_2O and HBSS for 18 h as described in Section 2.
[a] $P < 0.05$ compared with the initial inoculum.

Table 5
Resistance to exposure to acid for 24 h after pre-adaptation in salt and sucrose

Pre-adaptation condition[a]	Number of viable bacteria after 24 h		% of viability
	Without acid	With acid (pH 2.2)	
H_2O	$3.0 \pm 0.3 \times 10^6$	$2.6 \pm 0.4 \times 10^6$	87
HBSS	$2.8 \pm 0.4 \times 10^6$	$7.4 \pm 0.4 \times 10^5$	26.5[b]
NaCl in H_2O	$2.4 \pm 0.3 \times 10^6$	$3.4 \pm 0.2 \times 10^5$	14.2[b]
$CaCl_2$ in H_2O	$2.9 \pm 0.4 \times 10^6$	$2.2 \pm 0.3 \times 10^6$	76
$MgSO_4$ in H_2O	$2.8 \pm 0.2 \times 10^6$	$2.4 \pm 0.5 \times 10^6$	86
KCl in H_2O	$2.7 \pm 0.4 \times 10^6$	$6.1 \pm 0.3 \times 10^5$	22.6[b]
Sucrose in H_2O	$3.1 \pm 0.3 \times 10^6$	$7.1 \pm 0.3 \times 10^5$	23[b]

Initial inoculum: $3.4 \pm 0.3 \times 10^6$. The results are mean ± S.D. of three separate experiments.
[a]The concentrations of salt represent the concentrations present in HBSS. NaCl, 10.3 mg ml^{-1}; $CaCl_2$, 0.186 mg ml^{-1}; $MgSO_4$, 0.2 mg ml^{-1}; KCl, 11.5 mg ml^{-1}. Bacteria were grown in 7H9 broth, pH 6.8, then harvested and pre-adapted in H_2O, HBSS and H_2O supplemented with NaCl, $CaCl_2$, $MgSO_4$, KCl and sucrose at pH 6.8. After the pre-adaptation period, bacteria were exposed to acidic conditions.
[b]$P < 0.05$ compared with exposure to H_2O.

For this study, we developed an in vitro assay to determine if *M. avium* can tolerate the conditions typical of a normal fasting stomach, since the intestinal route is the primary route of *M. avium* infection in AIDS patients [4,5]. We found that *M. avium*, independent of the growth phase, is able to resist exposure to pH 2.2 for 2 h, although if the incubation period is prolonged up to 24 h, the viability of the bacteria decreased significantly. Studies with other enteric pathogens had shown that acid tolerance is dependent on the growth phase of the organisms and is significantly increased when the bacterium is grown to the stationary rather than exponential phase [8–10]. Maximal acid tolerance of *Salmonella typhimurium, Shigella flexneri* and *Listeria monocytogenes* is observed when the bacteria are pre-adapted by exposure to mild acidic conditions for a period of time [9,10,17]. In our experiments, acid resistance of *M. avium* was observed without pre-adaptation to acid. This finding either indicates that the bacterial cell wall may resist acid without the synthesis 'de novo' of proteins, lipid or carbohydrate or that the synthesis or incorporation of glutamate, as described for *E. coli* and *Shigella*, confers protection [18,19]. Our observation that inhibition of protein synthesis prior to and during acid exposure had no effect on acid resistance supports these hypotheses.

Incubation of *M. avium* under acidic conditions for 24 h, however, demonstrated that mechanisms other than cell wall natural resistance may be important in the ability to resist acidic conditions. A significant difference in acid resistance under prolonged exposure was observed between the exponential and stationary growth phase. Similar to enteric bacteria, *M. avium* grown to the stationary phase was more tolerant of acidic pH.

Small and colleagues [10] had demonstrated that the association of acid tolerance in *Shigella* and stationary growth phase is linked to the upregulation of the *rpoS* gene expression. This gene encodes a sigma factor regulated by environmental stress. Whether a sigma factor is upregulated on *M. avium* grown to the stationary phase is currently unknown, but evidence exists for a *rpoS* homolog, *sigE* in mycobacteria, which has been shown to be regulated by environmental factors [20].

Our findings that *M. avium* resistance to pH 2.2 was significantly increased when *M. avium* was incubated in water (an environmental habitat for the bacterium) [3] for 18 h and not when bacteria were exposed to an isoosmolar solution containing salts and glucose suggests a potential effect of some of the components of HBSS on gene regulation. Exposure to acid or anaerobiosis has been shown to increase *E. coli* and *S. flexneri* tolerance to acid even in the absence of *rpoS* expression [10,21]. Our subsequent studies demonstrated that isoosmolarity was likely to be the condition associated with impaired resistance of *M. avium* after 24 h, which confirmed the initial hypothesis that acid resistance may be a consequence of the presence of a common gene regulator in *M. avium*. Previous work in our laboratory has shown that environmental conditions can significantly alter the ability of *M. avium* to enter intestinal epithelial cell lines [14] and intestinal mucosa [22]. It is plausible to hypothesize that pre-incubation in hyposmolar conditions (water) would result in the triggering of mechanisms aimed at protecting the bacterium, such as closure of membrane porins and synthesis of lysine and glutamine, whereas incubation in isoosmolar conditions (HBSS) would not pre-adapt the bacterium to a subsequent acid exposure. The exact role that osmolarity, or specific ions, as well as other environmental factors such as low oxygen tension play in *M. avium* gene regulation are currently being investigated in our laboratory.

The findings of this work indicate that *M. avium* is extremely resistant to conditions found in the stomach both for short or long periods of time. This characteristic of the organism certainly contributes to the virulence and the ability to colonize the intestinal tract. Further studies will aim to determine the effect of environmental factors on acid resistance.

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