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Pasteurized Whole Milk Confers Reduced Susceptibilities to the Antimicrobial Agents Trimethoprim, Gatifloxacin, Cefotaxime and Tetracycline via the *marRAB* Locus in *Escherichia coli*

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SUMMARY

We inoculated pasteurized whole milk with *E. coli* strains GC4468 (intact *marRAB* locus), JHC1096 (Δ *marRAB*), or AG112 (Δ *marR*), and incubated each overnight at 37°C. All strains were then recovered from the milk cultures, and susceptibilities to antimicrobial agents were determined by the E-test strip method (CLSI). Cells of strain GC4468, prior to culturing in milk, were susceptible to trimethoprim, gatifloxacin, cefotaxime and tetracycline. After culturing GC4468 in pasteurized milk, however, the MICs increased 1.4-fold for trimethoprim ($P \leq 0.05$), 1.5-fold for gatifloxacin ($P \leq 0.05$), 2.0-fold for cefotaxime ($P = 0.008$), and 1.4-fold for tetracycline ($P \geq 0.05$). After culturing GC4468 on milk count agar the MICs were enhanced 3.4-fold for trimethoprim ($P \leq 0.05$), 10-fold for gatifloxacin ($P = 0.001$), 7.1-fold for cefotaxime ($P = 0.011$), and 40.5-fold for tetracycline ($P = 0.074$), but exhibiting tetracycline resistance with a mean MIC of 74.7 ± 18.47 μ g/ml (CLSI). The MICs of the antimicrobial agents for JHC1096 cells after culturing in pasteurized whole milk were indistinguishable ($P \geq 0.05$) from baseline MICs measured before culturing in the same type of milk. Thus, *E. coli* cells harboring the *marRAB* locus exhibit reduced susceptibilities to multiple antimicrobial agents after culturing in pasteurized whole milk.

Infectious diseases caused by bacteria that are resistant to antimicrobial agents are a serious public health concern (Levy & Marshall, 2004; Barbosa & Levy, 2000b; Neu, 1992). Members of the Enterobacteriaceae family of bacteria are causative agents of infectious disease, and their resistance to antimicrobial agents compromises chemotherapeutic efforts (Paterson, 2002). Antimicrobial agents are used in agriculture for the treatment of infection, prophylaxis and growth promotion (Levy & Marshall, 2004; Levy, 2002).

Bacterial resistance to antimicrobial agents may be of clinical significance due to dissemination of pathogenic bacteria through a population of food animals (McDermott *et al.*, 2002; Angulo *et al.*, 2004; Silbergeld *et al.*, 2008). In isolated instances, outbreaks of food-borne infectious disease from contaminated milk and occurrences of milk-derived isolates of members from the Enterobacteriaceae family of bacteria have been studied (Gillespie *et al.*, 2003). Although relatively well-studied within dairy cattle (Hershberger *et al.*, 2005), bacteria that are resistant to antimicrobial agents in other dairy farm environments, such as soil (Burgos *et al.*, 2005), water (Biyela *et al.*, 2004) and milk (Makovec & Ruegg, 2003) are less well-characterized. Dissemination mechanisms for drug resistant bacterial pathogens within dairy farms are also poorly understood (Hershberger *et al.*, 2005).

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The *marRAB* locus is a well studied genetic element in *Escherichia coli* and mediates bacterial resistance to multiple antimicrobial agents such as β -lactams, chloramphenicol, quinolones, and tetracycline (Randall & Woodward, 2002). Several organic compounds are known to modulate resistance to antimicrobial agents via the *marRAB* locus (Aleksun & Levy, 1999). Recently, milk-containing foods were shown to modulate the *marRAB* locus (Rickard *et al.*, 2004); the effects of milk, however, were not directly examined. Thus, we tested the hypothesis that milk confers reduced susceptibilities to antimicrobial agents in *E. coli* cells harboring the *marRAB* locus. The objective of this study was to examine the relationship between the culturing of *E. coli* in milk and the subsequent development of resistance to antimicrobial agents. Here, we found that upon culturing in pasteurized milk, antimicrobial agent-sensitive *E. coli* becomes less susceptible to multiple antimicrobial agents, a phenotype conferred by the *marRAB* locus.

Materials and Methods

Bacterial strains

E. coli K-12 bearing a deletion of the *marRAB* operon (strain JHC1096), was a generous gift from Dr. Jean Greenberg (Harvard University, MA) (Greenberg *et al.*, 1991). The *E. coli* strain harboring a deletion of *marR* (strain AG112) (George & Levy, 1983; Moken *et al.*, 1997), and a wild-type strain harboring an intact *marRAB* locus (strain GC4468) (Oethinger *et al.*, 1998; Carlioz & Touati, 1986) were generous gifts from the laboratory of Dr. Stuart Levy (Tufts University, MA).

Growth determination in Luria-Bertani broth and milk

Cells JHC1096, AG112, and GC4468 were grown overnight at 37 °C while shaking in Luria-Bertani (LB) broth (Difco), which is routinely used for maintenance and propagation of *E. coli* (Luria *et al.*, 1960). Then 20 μ l of culture was added to 20 ml fresh LB broth and grown to the mid-log phase of growth (O.D.₆₀₀ = 0.35). Next, 10 μ l of the mid-log culture was used to inoculate 10 ml of fresh LB broth or commercially available pasteurized whole milk. This procedure was repeated as above except that the LB broth and the pasteurized whole milk each contained 5 mM Salicylate. The cultures were incubated while shaking at 37 °C for the times indicated in Fig. 1 and Fig. 2. Samples (10 μ l) of the cultures were removed at the time points indicated, plated onto MacConkey agar, and incubated for 12 h. Colony forming units (CFU/ml) were then determined. The means plotted were the results of 4 independent experiments, each in triplicate.

Antimicrobial susceptibility testing

Prior to antimicrobial susceptibility testing via the E-Test assay using current CLSI guidelines (2006), *E. coli* cells JHC1096, AG112, and GC4468 were treated with LB broth (Difco) with and without 5 mM salicylate, commercially available pasteurized whole milk, or milk count agar (OXIOD) in the following manner: *E. coli* strains JHC1096, AG112 and GC4468 were inoculated in LB Broth (Difco), incubated overnight at 37°C with aeration and then used to inoculate refresher cultures in LB broth (Difco) for growth to the mid-log phase (O.D.₆₀₀ $\approx$ 0.35). The mid-log cells were used for incubation in LB broth (Difco) (with and without 5 mM salicylate) at 37 °C with aeration for 8 h or for incubation in commercially available pasteurized whole milk at 30 °C with aeration for 8 h. An additional 10 ml of pasteurized whole milk was inoculated with 10 μ l of sterile distilled water as a negative control. For the treatment in LB broth (Difco) with or without 5 mM salicylate, a total of 50 μ l of each culture was plated onto LB agar medium (Difco) plates and incubated overnight at 37 °C. For the treatment in pasteurized whole milk, a total of 50 μ l of each culture was plated onto either LB agar (Difco) or milk count agar (OXIOD) and incubated at 30 °C for 24 h.

For the antimicrobial agent susceptibility measurements, three single colonies of each strain were randomly picked from each of the plates, resuspended in 1 ml per colony of 0.85% NaCl and each immediately spread uniformly onto cation-adjusted Muller-Hinton II Agar (Difco) plates or Milk Count Agar. A portion of the 0.85 % NaCl resuspension (0.1 ml) was plated onto LB agar, and cells were identified by BD BBL Crystal Enteric/Nonfermentor Identification Kits (Becton Dickinson Microbiology Systems, Cockeysville, MD) according to the manufacturer specifications. E-Test strips (AB Biodisk) were placed on the surface as per the manufacturer, and the plates were incubated at 37 °C for 16 h. The MICs were read and interpreted according to current CLSI guidelines (2006). The means $\pm$ SDs (ranges) were the result of three independent experiments, each in triplicate, $n = 3$. Break points for susceptibility interpretations were MICs $\leq 8 \mu\text{g/ml}$ for trimethoprim, $\leq 2 \mu\text{g/ml}$ for gatifloxacin, $\leq 8 \mu\text{g/ml}$ for cefotaxime, and $\leq 4 \mu\text{g/ml}$ for tetracycline. The MIC break point for resistance to tetracycline was $\geq 16 \mu\text{g/ml}$.

Statistics

All data represent the mean plus or minus the standard deviation and range of at least three independent experiments ($n = 3$) conducted under standardized conditions. The nonparametric Holm-Sidak method was used to determine the significance of mean MIC data compared to mean baseline MICs within each strain. Results were considered significant at $P \leq 0.05$.

Results and Discussion

Baseline determinations of antimicrobial susceptibilities

Prior to culturing *E. coli* strains GC4468 (intact *marRAB* locus), JHC1096 (*marRAB* deleted) AG112 (*marR* deleted) in pasteurized whole milk or milk count agar, the minimal inhibitory concentrations (MICs) were determined by the standard E-test method (2006). All three *E. coli* strains were susceptible to the antimicrobial agents trimethoprim, gatifloxacin, cefotaxime and tetracycline (Table 1). These antimicrobial agents represent distinct drug classes and have different mechanisms of actions (McDermott *et al.*, 2003). These agents also have significance in clinical therapy, such as trimethoprim (Juckett, 1999; Casewell *et al.*, 2003), gatifloxacin (Lutsar *et al.*, 1999), and cefotaxime (Wittmann *et al.*, 1997) or significance in agriculture for the purposes of growth promotion or prophylaxis in dairy calves, such as tetracycline (Constable & Morin, 2002).

Antimicrobial susceptibilities after culturing in pasteurized milk and milk count agar

We found that after culturing *E. coli* GC4468 (intact *marRAB* locus) in pasteurized milk, the antimicrobial agent MICs increased by 1.4-fold for trimethoprim ($P = 0.05$), by 1.5-fold for gatifloxacin ($P \leq 0.05$), and by 2.0-fold for cefotaxime ($P = 0.008$), as shown in Table 1. The 1.4-fold increase in mean MIC for tetracycline was not significantly different in GC4468 ($P \geq 0.05$). The mean MIC in AG112 (*marR* deleted) increased ($P = 0.003$) by 2.8-fold for trimethoprim, but not for gatifloxacin, cefotaxime, and tetracycline ($P = 0.10$). The MICs for the same antimicrobial agents in *E. coli* AG112 were not significantly different ($P \geq 0.05$) compared to the MIC values obtained prior to culturing in milk and milk count agar, implying that the *marRAB* locus in AG112 was already intrinsically induced, as expected, and that another inducer (milk) would not further enhance susceptibility. The MICs of the antimicrobial agents tested in this study (ranged between 0.01 and 0.017 $\mu\text{g/ml}$) for cells of JHC1096 (*marRAB* locus deleted) after culturing in pasteurized whole milk were indistinguishable ($P \geq 0.05$) from the MICs determined prior to culturing (Table 1) in the same type of milk.

As shown in Table 1, we found that after culturing *E. coli* GC4468 on milk count agar, the antimicrobial agent MICs increased by 3.4-fold for trimethoprim ($P = 0.036$), by 10-fold for gatifloxacin ($P = 0.001$), by 7.1-fold for cefotaxime ($P = 0.011$), and by 40.5-fold for

tetracycline ($P = 0.074$) compared to the mean MICs derived prior to culturing on milk count agar. Interestingly, GC4468 cells on milk count agar with a mean MIC 74.67 $\mu\text{g/ml}$ are considered resistant to tetracycline, if not susceptible. As expected, cells of AG112 (*marR* deleted) were not less susceptible to the antimicrobial agents tested compared to baseline MICs ($P \geq 0.05$), regardless of whether milk or milk count agar was used, further implying that within induced cells, susceptibilities are not further reduced. For instance, AG112 cells were considered tetracycline resistant regardless of the variable tested (Table 1), as would be expected if the *marRAB* locus was induced. The MICs of the antimicrobial agents for JHC1096 cells (ranging between 0.01 – 0.017 $\mu\text{g/ml}$) after culturing in pasteurized whole milk or milk count agar were indistinguishable ($P \geq 0.05$) from the MICs determined prior to culturing (Table 1) in the same milk-type, except for an observed 5-fold increase in the MIC of gatifloxacin ($P < 0.05$) for JHC1096 cultured on milk count agar (but not pasteurized milk) compared to the baseline MIC in gatifloxacin. Prior to culturing in milk or milk agar, *E. coli* strains GC4468 and JHC1096 were susceptible to all antimicrobial agents tested. The reductions in antimicrobial agent susceptibilities in milk were more pronounced in GC4468 after sub-culturing on milk count agar compared to sub-culturing in pasteurized milk. We conclude that culturing in pasteurized milk or milk agar, *E. coli* cells harboring an intact *marRAB* locus become less susceptible to multiple antimicrobial agents.

Measurement of antimicrobial susceptibilities with salicylate

As a positive control experiment for the induction of the *marRAB* locus, the antimicrobial agent MICs were determined in 5mM salicylic acid, a well-known *marRAB* locus inducer (Aleksun & Levy, 1999). As shown in Table 1, in the presence of salicylate, the MICs increased for trimethoprim ($P = 0.01$) and cefotaxime ($P = 0.008$) in GC4468 cells. AG112 cells (*marR* deleted) with or without salicylate had tetracycline MICs of 213 and 256 $\mu\text{g/ml}$, respectively, while JHC1096 cells (*marRAB* locus deleted) remained susceptible to tetracycline and the other antimicrobial agents tested, consistent with the known *marRAB* locus resistance properties (Aleksun & Levy, 1999).

The observation that the antimicrobial agent susceptibilities were unchanged in the *E. coli* strain that lacks MarR and which constitutively expresses MarA, a multiple antibiotic resistance activator (Hachler *et al.*, 1991; Barbosa & Levy, 2000a), confirms the role of the *marRAB* locus in conferring reduced susceptibilities to antimicrobial agents by milk in GC468 cells, which possess an intact *marRAB* locus. The component within milk that modulates the *marRAB* locus remains to be identified. Although chromosomal elements are directly involved in the reduced antimicrobial susceptibilities observed here, we are nonetheless not able to definitively rule out the possibility that extra-chromosomal elements (i.e., plasmids, etc) may be involved in naturally-derived environmental or agriculture bacterial isolates (Burgos *et al.*, 2005). Future studies are necessary to distinguish between these alternatives. Along these lines, we are presently examining the antimicrobial agent susceptibility profiles for previously published *E. coli* isolates from dairy soil (Burgos *et al.*, 2005) and from dairy water for a new panel of antimicrobial agents.

Growth in LB medium and milk

The growth of *E. coli* strain AG112 cells reached higher growth plateaus when compared to those of GC4468 and JHC1096 strains in both LB broth and in milk (see Figure 1 and Figure 2). All three strains, however, reach higher growth plateaus in LB broth than in milk. Our investigation of the relationship of *E. coli* growth indicates that the total growth yield in LB broth is better than that in liquid milk while the antimicrobial agent susceptibilities are reduced, which is consistent with the contention that milk itself, and not growth in milk, is the factor conferring the reduced susceptibilities that we observe here.

In summary, pasteurized milk or milk agar media confers reduced susceptibilities to multiple antimicrobial agents in *E. coli*. Our studies provide evidence to support the contention that the mode of acquisition of reduced susceptibilities to antimicrobial agents by *E. coli* in pasteurized milk is due to modulation of the *marRAB* locus. We examined *E. coli*, which is a model bacterium and member of the Enterobacteriaceae family of bacteria, because of its relevance to physiology, its biomedical role as a causative agent of infectious disease and its antimicrobial agent resistance properties, which in turn compromise therapy (Sanders & Sanders, 1992; Alekshun & Levy, 2006; Baquero *et al.*, 1998). *E. coli* microorganisms have profound significance as a tool for molecular biology, microbial genetics, and as indicators of environmental sewage contamination, plus pathogenic *E. coli* variants have considerable clinical importance (Neidhardt, 1996). Because of their demonstrated transferability of antimicrobial agent resistance determinants among bacteria of distinct species (Leverstein-van Hall *et al.*, 2003; Neu, 1984; Nord, 1993), human consumption of raw milk contaminated with multidrug resistant microorganisms may contribute to the dissemination of multidrug resistant bacteria in agriculture and could be a source of potentially untreatable zoonotic infectious disease (Angulo *et al.*, 2004; McEwen & Fedorka-Cray, 2002; Tollefson *et al.*, 1999). Lastly, because the *marRAB* locus is present in other members of the Enterobacteriaceae family of bacteria, such as *Enterobacter*, *Klebsiella*, *Shigella*, and *Salmonella* (Cohen *et al.*, 1993), the findings presented here have relevance to these medically important microorganisms and their relationships with milk.

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A

B

Fig. 1. Effects of salicylate on bacterial growth (CFU) in LB broth

E. coli cells of strains AG112 (filled circles), JHC1096 (empty circles) and GC4468 (filled triangles) were incubated in LB broth without inducer (i.e., no salicylate), panel A, or in LB broth with inducer (5mM salicylate), panel B. See methods for details.

A

B

Fig. 2. Effects of salicylate on bacterial growth (CFU) in milk

E. coli cells of strains AG112 (filled circles), JHC1096 (empty circles) and GC4468 (filled triangles) were incubated in pasteurized milk without inducer (i.e., no salicylate), panel A, or in pasteurized milk containing inducer (5mM salicylate), panel B. See methods for details.

Table 1
Minimal inhibitory concentrations of antimicrobial agents for *E. coli* in pasteurized milk, milk count agar or salicylate.

Antimicrobial Agent MICs (µg/ml) [‡]					
<i>E. coli</i> Strains	Variables	Trimethoprim	Gatifloxacin	Cefotaxime	Tetracycline
GIC4468 (wild-type <i>marRAB</i>)	Baseline MIC	0.33 ± 0.046 (0.3 – 0.38)	0.01 ± 0.001 (0.01 – 0.012)	0.053 ± 0.003 (0.05 – 0.055)	1.83 ± 0.288 (1.5 – 2.0)
	Milk	0.46 ± 0.069 [*] (0.38 – 0.5)	0.0147 ± 0.0023 [*] (0.012 – 0.016)	0.104 ± 0.018 ^{***} (0.094 – 0.125)	2.5 ± 0.5 (2.5 – 3.0)
	Milk Count Agar	1.12 ± 0.44 [*] (0.75 – 1.6)	0.10 ± 0.019 ^{**} (0.09 – 0.125)	0.38 ± 0.125 [*] (0.25 – 0.5)	74.67 ± 18.47 (64 – 96)
	Salicylate	0.5 ± 0 [*] (0.5 – 0.5)	0.021 ± 0.0017 (0.02 – 0.023)	0.104 ± 0.018 ^{***} (0.094 – 0.125)	2.17 ± 0.288 (2.0 – 2.5)
	Baseline MIC	0.25 ± 0 (0.25 – 0.25)	0.0126 ± 0.0006 (0.012 – 0.013)	0.064 ± 0 (0.064 – 0.064)	1.67 ± 0.288 (1.5 – 2.0)
JHC1096 (deleted <i>marRAB</i>)	Milk	0.35 ± 0.046 (0.3 – 0.38)	0.017 ± 0.0023 (0.016 – 0.02)	0.061 ± 0.005 (0.055 – 0.064)	2 ± 0 (2.0 – 2.0)
	Milk Count Agar	0.42 ± 0.069 (0.38 – 0.5)	0.063 ± 0.0057 ^{***} (0.06 – 0.07)	0.0447 ± 0.004 (0.04 – 0.047)	1.42 ± 0.144 (1.25 – 1.5)
	Salicylate	0.25 ± 0.128 (0.125 – 0.38)	0.011 ± 0.0017 (0.008 – 0.012)	0.042 ± 0.0087 (0.032 – 0.047)	1.67 ± 0.288 (1.5 – 2.0)
	Baseline MIC	0.34 ± 0.04 (0.3 – 0.38)	0.017 ± 0.002 (0.016 – 0.02)	0.0997 ± 0.023 (0.08 – 0.0125)	213.33 ± 36.95 (192 – 256)
	Milk	0.916 ± 0.144 ^{***} (0.75 – 1.0)	0.037 ± 0.0086 (0.032 – 0.047)	0.147 ± 0.0375 (0.125 – 0.19)	256 ± 0 (256 – 256)
AG112 (deleted <i>marR</i>)	Milk Count Agar	0.75 ± 0 (0.75 – 0.75)	0.069 ± 0.009 (0.064 – 0.08)	0.25 ± 0 (0.25 – 0.25)	256 ± 0 (256 – 256)
	Salicylate	0.75 ± 0 (0.75 – 0.75)	0.037 ± 0.0086 (0.032 – 0.047)	0.115 ± 0.018 (0.094 – 0.125)	256 ± 0 (256 – 256)

[‡] MIC values (CLSI) are presented as the mean ± SD (range), n = 3. See methods for details.
^{*} P ≤ 0.05,
^{**} P ≤ 0.01 and

P ≤ 0.001 compared to baseline MICs for the same *E. coli* strain.