APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2007, p. 211–217 Vol. 73, No. 1
0099-2240/07/$08.00+0 doi:10.1128/AEM.00578-06

Environmental Stress and Antibiotic Resistance in Food-Related Pathogens[▽]

M. Ann S. McMahon,[1] Jiru Xu,[2] John E. Moore,[2] Ian S. Blair,[1] and David A. McDowell[1]*

Food Microbiology Research Group, University of Ulster, Shore Road, Newtownabbey, Northern Ireland, United Kingdom,[1] and Northern Public Health Laboratory Service, Belfast City Hospital, Lisburn Road, Belfast, Northern Ireland, United Kingdom[2]

Received 10 March 2006/Accepted 30 October 2006

This study investigated the possibility that sublethal food preservation stresses (high or low temperature and osmotic and pH stress) can lead to changes in the nature and scale of antibiotic resistance (ABR) expressed by three food-related pathogens (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*). The study found that some sublethal stresses significantly altered antibiotic resistance. Incubation at sublethal high temperature (45°C) decreased ABR. Incubation under increased salt (>4.5%) or reduced pH (<5.0) conditions increased ABR. Some of the pathogens continued to express higher levels of ABR after removal of stress, suggesting that in some cases the applied sublethal stress had induced stable increases in ABR. These results indicate that increased use of bacteriostatic (sublethal), rather than bactericidal (lethal), food preservation systems may be contributing to the development and dissemination of ABR among important food-borne pathogens.

Environmental stress can be defined as an external factor that has an adverse effect on the physiological welfare of bacterial cells, leading to reduction in growth rate, or in more extreme circumstances, to inhibition and/or death, at individual cell or population levels. Examples of such bacteriostatic or bactericidal stresses include extremes of temperature, pH, osmotic pressure, depletion of nutrients (6), and the presence of toxic or inhibitory compounds, including antibiotics (26). Many modern food preservation processes aim to extend the lag phase of bacteria present in food by the application of one or more environmental stresses to slow or prevent bacterial growth. However, absence of growth does not necessarily mean absence of metabolic and/or genetic activity, and stress-inhibited bacteria act to reduce the impact of environmental stresses by making phenotypic and genotypic adaptations (34). Examples of phenotypic alteration include expression of protective shock proteins, which provide resistance to subsequent challenge with the same stress (stress hardening) (31) and can also lead to cross-protection against a range of apparently unrelated challenges, including resistance to antibiotics (5, 18, 31, 36). Genotypic alterations include stress-induced genetic plasticity where microorganisms increase their rates of random mutagenesis and intra- and intercellular transfer of genes (16, 36). These alterations increase population diversity, increasing the chances that at least some cells may survive and grow under the conditions of stress.

It is currently believed that bacterial cells sense the actions of antibiotics as just another form of environmental stress (26) and that sublethal concentrations of antibiotics induce stress hardening (8) and cross-protection (15). Conversely environmental stresses can induce resistance to antibiotics by a range of means.

Many environmental stresses, including detergents, organic solvents, dyes, food components, preservatives, and antibiotics, induce the *mar* (multiple antibiotic resistance) operon. This operon (3) regulates the expression of a large number of genes, including those coding for at least one broad-specificity efflux pump (the *arcAB* efflux pump) (30), which are more strongly expressed under conditions of environmental stress (23). This suggests a direct linkage between environmental stresses, such as those occurring in foods and the domestic environment (3), efflux pump expression, and the development of antibiotic resistance (ABR) (12, 14, 30).

Other mechanisms that have been associated with the development or expression of ABR include expression of specialized bacterial proteins, transfer of plasmids, or generation of genetic variability through mutation. Bishop et al. (7) indicated that the expression of bacterial lipocalin, a protein implicated in the adaptation of bacterial cells to environmental stress and the dissemination of ABR genes, is regulated by high osmolarity and starvation. The bacterial lipocalin gene (*blc*) is carried on multiple antibiotic resistance plasmids in some genes in *Enterobacteriaceae*, rendering them capable of transfer between and within bacterial species (21). As well as encouraging plasmid transfer, environmental stress can modulate plasmid numbers, enhancing resistance due to higher plasmid copy numbers. Such increases induce a range of protective responses, including expression of bactericidal exoproteins to suppress the growth of competing bacteria, and mutagenesis, including the induction of the SOS response (26, 36).

Under nonstress conditions, DNA repair systems operate very efficiently to sustain population homogeneity with strict genetic conservation and fidelity. Rates of expression of classical mutations are low, only occurring when random genetic errors evade the DNA repair systems (11). Under adverse (stress) conditions, population homogeneity is less desirable

* Corresponding author. Mailing address: Food Microbiology Research Group, 15J09b, University of Ulster, Shore Road, Newtownabbey BT37 0QB, Northern Ireland, United Kingdom. Phone: 44 28 90 366697. Fax: 44 28 90 368811. E-mail: da.mcdowell@ulster.ac.uk.

▽ Published ahead of print on 1 December 2006.

TABLE 1. Selected sublethal environmental stresses

Organism or condition	$MHB-_{temp}$ (°C)	$MHB+_{temp}$ (°C)	MHB_{NaCl} (%)	MHB_{pH}
E. coli	10	45	4.5	4.5
S. aureus	21	45	12	5.0
S. enterica serovar Typhimurium	10	45	4.5	4.0
Control	37	37	0	7.4

and consistency in DNA replication is not as important. In fact, some reduction in consistency may be essential to produce a more heterogeneous population, some members of which possess the new genes or gene combinations to better survive the adverse conditions. This process has been described as adaptive or directed mutation. The mechanisms underlying adaptive/directed mutation include stress-induced errors during DNA synthesis, suppression of normal DNA repair checking and repair mechanisms, transient hypermutability, gene amplification, and stress-induced recombination processes (26).

Environmental stresses such as oxidative stress, pH extremes, anaerobiosis, heat shock, osmotic shock, and starvation are reported to induce genomic reorganization or mutation (16) in bacterial cells, and such mechanisms of adaptive mutation have been associated with the development of ABR (11).

The aim of this work was to determine if environmental stresses of the types typically used in food preservation influence the development or expression of antibiotic resistance in food-related pathogens.

MATERIALS AND METHODS

Organisms. Wild-type strains of *Salmonella enterica* serovar Typhimurium DT 104 ($n = 4$) were isolated by the Northern Ireland Public Health Laboratory Service from raw mussels (st11 and st12), chicken (st16), and egg mix/fried rice (st17). *Escherichia coli* wild-type strains ($n = 4$), and *Staphylococcus aureus* ($n = 4$) were isolated from surface swabs taken from commercial food kitchens. *Escherichia coli* strains were isolated and identified to the species level using Chromocult coliform agar (Merck). Identification was confirmed using API 20E (Bio Merieux). *Staphylococcus aureus* strains were isolated on Baird Parker agar (Oxoid), and presumptive colonies were confirmed using the coagulase test (Dryspot Staphytect Plus; Oxoid) and API Staph (bioMerieux).

Determination of sublethal stress (NaCl, pH, high and low temperature). Each wild-type strain of *S. enterica* serovar Typhimurium (st11, st12, st16, and st17), *E. coli* (ec1, ec2, ec3, and ec5), and *S. aureus* (sa3, sa4, sa5, and sa6) was subcultured into Mueller-Hinton broth (MHB; Oxoid) at 37°C for 17 h. This medium has been recommended for the culture and assessment of antibiotic resistance in stressed bacteria (10). Cells were harvested by centrifugation ($8,000 \times g$ for 10 min) and washed twice in sterile Ringer's solution (Oxoid). The washed cells were resuspended and diluted in Ringer's solution to form standard inocula with an optical density at 600 nm (OD_{600}) of 0.1 ($\approx 10^8$ CFU/ml).

Duplicate aliquots of MHB (20 ml) were (i) supplemented with NaCl (BDH; Analar) to final concentrations of 3%, 4%, 4.5%, 5%, 5.5%, 6%, 12%, and 13% (wt/vol) (MHB_{NaCl}) (control, 0% additional NaCl); (ii) acidified with concentrated HCl (BDH) to final pH values of 5.5, 5.0, 4.5, 4.0, and pH 3.5 (MHB_{pH}) (control, pH 7.4); or (iii) equilibrated to suboptimal temperatures (i.e., 2°C, 3°C, 5°C, 10°C, and 21°C [$MHB-_{temp}$]) or superoptimal temperatures (40°C, 43°C, 44°C, 45°C, 46°C, 48°C and 50°C ± 1°C [$MHB+_{temp}$]) in temperature-controlled water baths (control, 37°C) and inoculated with 200 μl of one of the above standard inocula.

NaCl or acid-supplemented suspensions were incubated at 37°C for 24 h or until no increase was in observed in OD readings. High-temperature and low-temperature suspensions were incubated at the temperatures indicated until no increase was in observed in OD readings. The optical density of each culture was determined at 600 nm. The sublethal stress level was determined as the NaCl concentration, pH, or temperature at which a 75% reduction in OD_{600} of stressed cultures was observed compared to that of the control cultures indicated.

TABLE 2. Antibiotic usage in MIC determination

Organism	Antibiotics
E. coli	Amikacin, ceftriaxone, nalidixic acid
S. aureus	Gentamicin, oxacillin, erythromycin
S. enterica serovar Typhimurium	Amikacin, ceftriaxone, trimethoprim

Preparation of sublethal environmental stress broth cultures. Duplicate MHB aliquots (100 ml) were supplemented with NaCl to 4.5% or 12% (wt/vol); acidified with HCl to pH values of 5.0, 4.5, or 4.0; or preincubated at 10°C, 21°C, or 45°C to achieve selected sublethal environmental stress conditions. These aliquots, plus NaCl, pH, and temperature control samples as indicated above, were inoculated with the standard inocula (described above) as indicated in Table 1.

Stressed and control cultures were incubated until no increases in OD readings were recorded. Ten-milliliter aliquots of each culture were aseptically removed. Cells were harvested by centrifugation ($8,000 \times g$ for 10 min) and washed twice with sterile Ringer's solution (Oxoid). The washed cells were resuspended and diluted in Ringer's solution to form a standardized cell suspension (stressed and control cultures) of 10^5 to 10^6 CFU/ml approximately.

Determination of antibiotic susceptibility. (i) MIC determination with antibiotic stock solutions. Antibiotics (Sigma-Aldrich) were dissolved as per the manufacturer's recommendations to the following concentrations in MHB: amikacin, 60 and 40 μg/ml; ceftriaxone, 6 and 4 μg/ml; nalidixic acid, 60 and 40 μg/ml; gentamicin, 80 and 60 μg/ml; oxacillin, 8 and 6 μg/ml; erythromycin, 16 and 12 μg/ml; and trimethoprim, 6 and 4 μg/ml. Stock solutions were stored at −20°C and used within 5 days. Stock solutions were used to determine MICs for each standardized stressed and control cell suspension as indicated in Table 2.

(ii) MIC determination matrices. Unsupplemented MIC determination matrices were created as follows. Serial twofold dilutions of each antibiotic stock solution were prepared in MHB in sterile 96-well microdilution plates (Sarstedt; 12 columns by 8 rows) by the method of Jörgensen and Ferraro (17), thus allowing 12 columns of 7 twofold dilutions of each antibiotic per 96-well plate. The eighth well in each column contained MHB to form a sterility control and plate reader blank.

Stress-supplemented MIC (SSMIC) determination matrices were prepared in an identical manner, except that the MHB diluent was supplemented with NaCl or HCl to achieve the same conditions as those used in the initial preparation of the stressed cell suspensions: i.e., low pH (5.0, 4.5, or 4.0) and high NaCl concentration (4.5% or 12%).

Estimation of antibiotic susceptibility. (i) Maintained stress conditions. The antibiotic susceptibilities of standardized stressed and control cell suspensions were estimated under maintained stress conditions using the SSMIC determination matrices. Each standardized stressed-cell suspension (10 μl) was inoculated in triplicate into antibiotic dilution series in SSMIC determination matrices as indicated in Table 2. NaCl- and HCl-supplemented (SSMIC) plates were incubated in a humidified chamber at 37°C for 24 h before being read at 660 nm and examined using a Titertek Plus plate reader (ICN Biomedicals Ltd.). In each case, the MIC was determined as the lowest concentration of antibiotic at which the OD_{660} reading was <0.1. Maintenance of thermal stress was achieved by incubating MHB MIC matrices at stress temperatures (i.e., 10°C, 21°C, or 45°C) before and after inoculation with standardized stressed and control suspensions.

(ii) Poststress conditions and control (nonstress) conditions. Antibiotic susceptibility under poststress conditions and control (nonstress) conditions was estimated using the unsupplemented MIC determination matrices as follows. Each standardized stressed-cell suspension or control cell suspension (10 μl) was inoculated in triplicate into antibiotic dilution series in MIC determination matrices as indicated in Table 2. MIC plates were incubated in a humidified chamber at 37°C for 24 h as described above.

Modification of the Stokes method to determine the presence of hyperresistant subpopulations. Each stressed standardized cell suspension was applied to one-half of the surface of a Mueller-Hinton agar plate (Oxoid; 20 ml) using a sterile swab (two plates per sample). The corresponding control standardized cell suspension was applied to the other half of the same agar plate in the same manner. The plates were allowed to equilibrate at room temperature for 15 min prior to the application of the appropriate antimicrobial susceptibility disc to the center of the agar plate. *E. coli* suspensions were tested using discs containing 30 μg amikacin, 30 μg ceftriaxone, and 5 μg trimethoprim. *S. enterica* serovar

TABLE 3. Changes in MIC in environmentally stressed *E. coli* in the presence of applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5
Amikacin	20 (20)	15 (15)	20 (15)	10 (10)	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	+	++	+	0	0	++	+	+
Ceftriaxone	0.09 (0.09)	0.09 (0.09)	0.25 (0.25)	0.09 (0.09)	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	+++	+	++	+	+++	+++	+++	+++
Nalidixic acid	7.5 (7.5)	7.5 (7.5)	5 (5)	7.5 (7.5)	−−−	−	−−	0	−−	0	−	0	+	+	++	+	0	+++	++	++

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in MIC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

Typhimurium was tested with discs containing 30 μg amikacin, 30 μg ceftriaxone, and 30 μg nalidixic acid. *S. aureus* cells were tested with discs containing 5 μg erythromycin, 10 μg gentamicin, and 5 μg oxacillin. (All antimicrobial susceptibility discs were purchased from Oxoid.) The plates were incubated at 37°C for 17 to 24 h. Plates were then examined for the development of zones of inhibition in each lawn growth around the central disc. Plates were also examined for the presence of hyperresistant colonies—isolated colonies that grew within the general zone of inhibition surrounding each disc. Such colonies were subcultured onto Mueller-Hinton agar, and confirmed as the inoculated species by appropriate biochemical tests. The MIC of standardized cell suspensions derived from such colonies was determined by the previously described 96-well broth microdilution method.

Statistical analysis. All MICs were determined in triplicate on three separate occasions. These data were analyzed as recommended for discrete values (2, 19). In addition, the presence or absence of significant differences ($P < 0.05$) between control and test data was examined by Mann-Whitney U analysis using SPSS 11.0.

RESULTS

The MICs of the antibiotics used against *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* before (control), during the application of, or after the removal of thermal, high-salt, and low-pH stresses were determined using the 96-well microdilution method and are shown in Tables 3 to 8. Statistically significant differences ($P < 0.05$) were established between control and treatment data using the appropriate median, mode, and Mann-Whitney U analysis.

Under conditions of continuing low-temperature stress, *E. coli*, *S.* enterica serovar Typhimurium, and *S. aureus* suspensions had lower MICs (of each of the three antibiotics used against each species) than unstressed control suspensions of these organisms (Tables 3, 4, and 5). The MICs for post-low-temperature-stressed suspensions of *E. coli* were similar to those observed in unstressed control suspensions (Table 6). Poststressed *S. enterica* serovar Typhimurium suspensions had lower MICs of ceftriaxone and trimethoprim than unstressed control suspensions (Table 7). Poststressed *S. aureus* suspensions had lower MICs of oxacillin; however, the MICs of gentamicin and erythromycin were similar to those observed in the unstressed control suspensions (Table 8).

Under conditions of continuing high-temperature stress, the MICs of the antibiotics applied to each species (i.e., *E. coli*, *S. enterica* serovar Typhimurium, and *S. aureus*) were lower than the MICs for (control) unstressed organisms (Tables 3 to 5).

The MICs of post-high-temperature-stressed suspensions of *S. aureus* were lower than the MICs of their unstressed control suspensions (Table 8). The MICs of post-high-temperature-stressed *S. enterica* serovar Typhimurium suspensions were similar to the MICs of the unstressed controls (Table 7). Under such conditions, high-temperature-stressed *E. coli* suspensions exhibited a similar response to ceftriaxone and nalidixic acid. However, under conditions of post-high-temperature stress, *E. coli* suspensions had an amikacin MIC that was lower than the MIC for the unstressed control (Table 6).

Under conditions of continuing high-salt or low-pH stress, the MICs of the antibiotic applied to each species were higher than the MICs for the unstressed control suspensions (Tables 3 to 5). Post-NaCl- or -acid-stressed *E. coli* suspensions had higher MICs of amikacin, ceftriaxone, and nalidixic acid than control suspensions (in many cases a greater than fourfold increase was observed) (Table 6).

Post-NaCl or post-acid-stressed *S. aureus* suspensions had higher MICs of gentamicin and erythromycin than control suspensions (Table 8).

Post-NaCl or post-acid-stressed *S. enterica* serovar Typhimurium suspensions had MICs that were similar to or less than those recorded for control suspensions (Table 7).

Modified Stokes method. The Stokes method did not demonstrate significant differences in zone sizes between test and control suspensions under any of the applied stresses or stress combinations. This method did, however, allow the observa-

TABLE 4. Changes in MIC in environmentally stressed *S. enterica* serovar Typhimurium in the presence of applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	11	12	16	17	11	12	16	17	11	12	16	17	11	12	16	17	11	12	16	17
Amikacin	15 (15)	20 (20)	20 (20)	20 (20)	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	+	0	0	0	++	++	++	++
Ceftriaxone	1 (1)	1.5 (1)	1 (0.75)	1 (1)	−−	−−−	−−	−−	−−	−−−	−−	−−	+	+	+	+	+++	++	+++	+++
Trimethoprim	3 (3)	3 (3)	3 (3)	3 (3)	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	++	++	++	−−	+++	+++	+++	+++

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in IC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

TABLE 5. Changes in MIC in environmentally stressed *S. aureus* in the presence of applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6
Gentamicin	2.5 (2.5)	2.5 (2.5)	2.5 (2.5)	1.9 (1.9)	−−	−−	−−	−−	−−	−−	−−	−−	+++	+++	+++	+++	+++	+++	+++	+++
Oxacillin	3 (3)	1 (1)	1 (1)	0.19 (0.19)	−−	−	−	0	−−−	−−−	−−−	−−	0	−	−	+++	−−−	−−−	−−−	−
Erythromycin	3 (3)	1 (1)	1 (1)	1 (1)	−−−	−	0	0	−−−	−−−	−−−	−−−	++	+++	++	+	++	+++	+++	+++

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in MIC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

tion and recovery of hyperresistant clones (i.e., colonies developing within the main zones of inhibition around the antibiotic disc), in a number of bacterium-stress-antibiotic combinations (Table 9). Some of these hyperresistant colonies occurred close to the antibiotic disc, suggesting that these colonies contained exceptionally resistant clones. The development of hyperresistant colonies was observed in all three species, but not all antibiotic-stress combinations. Such resistant clones were only recovered from treatments involving low-pH or high-NaCl stress. Of 25 sample clones randomly recovered for further analysis, 14 reverted to control MIC levels during subculture in the absence of environmental stress. However, the other 11 hyperresistant isolates continued to display higher MICs during repeated subculture (daily subculture for 72 h) in the absence of the sublethal environmental stress. Examples of the stable differences between parent strains and stress-induced stable hyperresistant mutants (SHRMs) for each of the species examined are presented in Table 10.

DISCUSSION

Antibiotic susceptibility in *E. coli*, *S. enterica* serovar Typhimurium, and *S. aureus* increased when these organisms were subjected to temperature stresses. A similar response has been reported in *Stenotrophomonas* spp. grown under high-temperature stress (29). Such increased susceptibility was maintained in post-high-temperature-stressed *S. aureus* cultures and post-low-temperature-stressed *S. enterica* serovar Typhimurium cultures. However, increased antibiotic susceptibility was not maintained in *E. coli* cultures when high- or low-temperature stress was removed or in *S. enterica* serovar Typhimurium and *S. aureus* cultures on removal of high- and low-temperature stresses, respectively.

These temporary changes may be due to transient alterations in the composition of the bacterial cell membranes or cell wall**.** Alterations in environmental temperature adversely change membrane fluidity, leading bacteria to make compensatory alterations in the degree of saturation of membrane fatty acids (4, 20, 27, 28) to reestablish ideal states of membrane fluidity. Loss of ideal states of membrane fluidity may have an impact on membrane receptor and signaling activities, reducing the rates and efficacy of binding of antibiotics (29, 35) and their import or export through bacterial membranes (27). On their return to optimum temperature conditions (i.e., removal of temperature stress), bacteria may rebalance the degree of saturation of membrane fatty acids (4, 20, 27, 28) to nonstress conditions, reestablishing optimum membrane structure and function, with a concomitant return to previously observed rates of ABR binding, import, and export. However, further work will be required to elucidate the processes by which thermal stress can induce persistent increases in antibiotic susceptibility. This study has, however, established significant differences between the patterns of changes in susceptibility induced by thermal stress and the patterns induced by pH and NaCl stresses.

In the presence of sublethal low-pH or high-NaCl stress, *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium displayed decreased susceptibility to antibiotics, with greater than fourfold increases in MIC observed in many cases. Significantly, such decreased susceptibility was maintained by *E. coli* and *S. aureus* (but not *S. enterica* serovar Typhimurium) after the stress was removed.

Viewing the MIC results in isolation does not allow identification of the possible mechanism underlying such reductions in susceptibility. The application of the modified Stokes method allowed the identification of clones growing close to the antibiotic disc, within the zone of "general" inhibition. Such SHRMs were observed in *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium subjected to low-pH and high-NaCl stress, but not in any of these organisms subjected to

TABLE 6. Change to MICs in environmentally stressed *E. coli* determined in the absence of the applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5	1	2	3	5
Amikacin	20 (20)	15 (15)	20 (15)	10 (10)	0	+	0	+	−−−	−−−	−−−	−−−	+	++	+	0	0	++	+	+
Ceftriaxone	0.09 (0.09)	0.09 (0.09)	0.25 (0.25)	0.09 (0.09)	0	0	−−	+	0	−−	−−	−−	+++	+++	+	++	++	+++	−	+++
Nalidixic acid	7.5	7.5	5	7.5	−	−	0	−	−	−	0	0	+	++	++	+	+	++	+++	++

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in MIC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

TABLE 7. Change to MICs in environmentally stressed *S. enterica* serovar Typhimurium determined in the absence of the applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	11	12	16	17	11	12	16	17	11	12	16	17	11	12	16	17	11	12	16	17
Amikacin	15 (15)	20 (20)	20 (20)	20 (20)	0	0	−−	0	0	−	−	−	−	−−	−−	−−	−	−−	−	−
Ceftriaxone	1 (1)	1.5 (1)	1 (0.75)	1 (1)	−	−−	−	−	−	0	−	−	−−−	−−	−−	−−−	0	−−	0	0
Trimethoprim	3 (3)	3 (3)	3 (3)	3 (3)	−−	−−	−−	−−	0	0	0	0	0	−	−−	−−	0	−	−	0

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in MIC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

high- or low-temperature stress. These observations suggest that any observed increases in MICs could be a stress response involving the whole population or the selection and outgrowth of a subpopulation of hyperresistant clones. In most cases, those stress systems which displayed hyperresistant colonies did not display significant changes in zone size. This observation demonstrates that stand-alone MIC analyses will not differentiate between changes in "whole-population resistance" and the outgrowth of small numbers of hyperresistant forms.

The mechanisms contributing to such stable whole-population resistance and hyperresistant clone formation may be due to long-term genotypic changes such as the following. (i) One change might be point mutations, which are known to lead to inactivation of target sites for antibiotic binding (21), leading to antibiotic inefficiency. (ii) A second change might be hypermutability: growth conditions affect bacterial mutation rate, generating heterogeneous populations with increased spontaneous mutation rates (hypermutable strains) and thus clones which may survive the stressful environmental conditions (25). Such hypermutable pseudomonad strains have been reported forming colonies within the zone of inhibition surrounding antibiotic discs (24). (iii) A third change might be adaptive or directed mutations. Adverse bacterial growth conditions have been reported to affect or increase the mutation rate (25, 31).

Temporary decreases in antibiotic susceptibility were also observed in sublethal low-pH and high-NaCl suspensions. *Salmonella enterica* serovar Typhimurium suspensions had decreased antibiotic susceptibility in the presence of the selective pressure (low pH or high NaCl); such decreased susceptibility was not maintained on removal of the stress. This transitory decrease in antibiotic susceptibility was also found in a proportion of *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium hyperresistant colonies. The mechanisms contributing to transient decreases in antibiotic susceptibility may be one of many short-term phenotypic changes, such as the following: (i) down-regulation of cell wall binding sites, (ii) gene amplification, and (iii) induction of shock protein. For the first mechanism, bacteria respond to environmental stresses (increased osmotic pressure, acid stress) by down-regulation of cell wall proteins such as penicillin binding proteins, reducing their sensitivity to and increasing their ability to survive in higher concentrations of antibiotic (9, 13, 32, 37). Such proteins may be up-regulated on removal of the environmental stress. With regard to gene amplification, decreases in antibiotic susceptibility been reported in bacteria grown in sublethal concentrations of antibiotic (1, 33), where the resultant hyperresistant phenotypes had increased copy numbers of antibiotic resistance genes. These hyperresistant phenotypes were unstable, and the basal susceptibility level was recovered on removal of the antibiotic from the medium. Finally, induction of shock proteins during acid and osmotic stress may provide cross-protection against the antibiotics used (5, 18, 22, 31, 36). Such proteins may be down-regulated on removal of the environmental stress, reversing this response.

Such transient stress responses are less likely to be of significance in the overall development and dissemination of antibiotic resistance, because the observed temporary decreases in susceptibility are unlikely to persist in consumed food to influence "in vivo" host-pathogen interactions. However, they reemphasize the extent to which global bacterial stress response to adverse environmental conditions can trigger cross-protection against seemingly unrelated challenges.

The stable or transitory nature of decreased antibiotic susceptibility in stressed pathogens such as *S. aureus*, *E. coli*, and *S. enterica* serovar Typhimurium indicates the importance of comprehensive susceptibility testing, where the subculturing and repeat susceptibility testing of hyperresistant colonies

TABLE 8. Change to MICs in environmentally stressed *S. aureus* determined in the absence of the applied sublethal environmental stress

Antibiotic	MIC (μg/ml) in control cultures for isolate[a]:				Stress type for isolate[b]:															
					Low temp				High temp				NaCl				pH			
	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6	3	4	5	6
Gentamicin	2.5 (2.5)	2.5 (2.5)	2.5 (2.5)	1.9 (1.9)	0	0	0	0	−−	−−	−−	−−	+++	+	++	+	+	+	0	0
Oxacillin	3 (3)	1 (1)	1 (1)	0.19 (0.19)	0	−−	−−	−−	−−−	−−	−−	0	++	0	−	−	−	−	−	0
Erythromycin	3 (3)	1 (1)	1 (1)	1 (1)	−−	0	0	+	−−−	−	−	−−	0	++	++	+	+	++	+	+

[a] Mode MIC ($n = 9$). Median values are given in parentheses.
[b] +, 1.5- to 2-fold increase in MIC ($P < 0.05$); ++, 2.1- to 4-fold increase in MIC ($P < 0.05$); +++, greater than 4-fold increase in MIC ($P < 0.05$); −, 1.5- to 2-fold decrease in MIC ($P < 0.05$); −−, 2.1- to 4-fold decrease in MIC ($P < 0.05$); −−−, greater than 4-fold decrease in MIC ($P < 0.05$); 0, no change ($P > 0.05$).

TABLE 9. Presence of hyperresistant colonies in control (unstressed) and stressed populations as determined by the modified Stokes method

Organism	Stress	Antibiotic	Presence of hyperresistant colonies
E. coli	Control	Amikacin	
		Ceftriaxone	
		Nalidixic acid	
	45 or 10°C	Amikacin	
		Ceftriaxone	
		Nalidixic acid	
	pH	Amikacin	
		Ceftriaxone	+
		Nalidixic acid	+
	NaCl	Amikacin	+
		Ceftriaxone	+
		Nalidixic acid	+
S. enterica serovar Typhimurium	Control	Ceftriaxone	
		Amikacin	
		Trimethoprim	
	45 or 10°C	Ceftriaxone	
		Amikacin	
		Trimethoprim	
	pH	Ceftriaxone	
		Amikacin	+
		Trimethoprim	
	NaCl	Ceftriaxone	+
		Amikacin	+
		Trimethoprim	+
S. aureus	Control	Oxacillin	
		Erythromycin	
		Gentamicin	
	45 or 21°C	Oxacillin	
		Erythromycin	
		Gentamicin	
	pH	Oxacillin	+
		Erythromycin	+
		Gentamicin	
	NaCl	Oxacillin	+
		Erythromycin	
		Gentamicin	

would provide a more accurate determination of antibiotic resistance.

Conclusion. This study demonstrates that some common food preservation processes (i.e., those which employ low pH or high NaCl to prevent or reduce pathogen growth rate) can lead to the development of populations or subpopulations of pathogens with decreased susceptibility to a range of currently used antibiotics. Such decreases in antibiotic susceptibility are maintained as long as the food preservation stress is maintained, and in some cases, even after the food preservation stress is removed, contributing to temporary or persistent ABR in all or part of the stressed bacterial population. This suggests that the increased use of bacteriostatic, rather than bactericidal, food preservation systems may be contributing to the development and dissemination of ABR among important food-related pathogens.

TABLE 10. Examples of differences in MICs between parent strains and SHRMs

Organism	Antibiotic	Stress type	MIC (μg/ml) for[a]:		MIC increase (fold)
			Parent	SHRM	
E. coli	Ceftriaxone	NaCl	0.09 (0.09)	75 (75)	833
	Naladixic acid	pH	7.5 (7.5)	37.5 (37.5)	5
S. enterica serovar Typhimurium	Trimethoprim	pH	1.6 (1.6)	6.25 (6.25)	4
S. aureus	Erythromycin	pH	3.1 (3.1)	6.25 (6.25)	2

[a] Mode MICs ($n = 3$). Median MICs are given in parentheses.

ACKNOWLEDGMENT

This study was supported by funding from the Research & Development Office, Department of Health, Northern Ireland: Infectious Disease—Recognized Research Group (RRG) 9.9.

REFERENCES

1. **Adams, J.** 2004. Microbial evolution in laboratory environments. Res. Microbiol. **155:**311–318.
2. **Aldridge, K. E., and W. D. Johnson.** 1997. A comparison of susceptibility results of the *Bacteriodes fragilis* group and other anaerobes by traditional MIC results and statistical methods. J. Antimicrob. Chemother. **39:**319–324.
3. **Alekshun, M. N., and S. B. Levy.** 1999. Alteration of the repressor activity of MarR, the negative regulator of the *Escherichia coli marRAB* locus, by multiple chemicals in vitro. J. Bacteriol. **181:**4669–4672.
4. **Alekshun, M. N., and S. B. Levy.** 2000. Bacterial drug resistance: responses to survival threats, p. 323–368. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, DC.
5. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
6. **Archer, D. L.** 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. Trends Food Sci. Technol. **7:**91–95.
7. **Bishop, R. E.** 2000. The bacterial lipocalins. Biochim. Biophys. Acta **1482:** 73–83.
8. **Blackburn, C. D., and A. R. Davies.** 1994. Development of antibiotic resistant strains for the enumeration of foodborne pathogenic bacteria in stored foods. Int. J. Food Microbiol. **24:**125–136.
9. **Bremer, E., and R. Krämer.** 2000. Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in bacteria, p. 79–97. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, DC.
10. **Butler, T., R. W. Frenck, R. B. Johnson, and R. Khakhria.** 2001. In vitro effects of azithromycin on *Salmonella typhi*: early inhibition by concentrations less than the MIC and reduction of MIC by alkaline pH and small inocula. J. Antimicrob. Chemother. **47:**455–458.
11. **Foster, P. L.** 2000. Adaptive mutation: implications for evolution. Bioessays **22:**1067–1074.
12. **Gilbert, P., and A. J. McBain.** 2003. Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. Clin. Microbiol. Rev. **16:**189–208.
13. **Gould, I. M., and F. M. MacKenzie.** 1997. The response of Enterobacteriaceae to beta-lactam antibiotics—'round forms, filaments and the root of all evil.' J. Antimicrob. Chemother **40:**495–499.
14. **Greenway, D. L. A., and R. R. England.** 1999. The intrinsic resistance of Escherichia coli to various antimicrobial agents requires ppGpp and sigma(s). Lett. Appl. Microbiol. **29:**323–326.
15. **Hastings, P. J., S. M. Rosenberg, and A. Slack.** 2004. Antibiotic-induced lateral transfer of antibiotic resistance. Trends Microbiol. **12:**401–404.
16. **Jolivet-Gougeon, A., S. David-Jobert, Z. Tamanai-Shacoori, C. Menard, and M. Cormier.** 2000. Osmotic stress-induced genetic rearrangements in *Escherichia coli* H10407 detected by randomly amplified polymorphic DNA analysis. Appl. Environ. Microbiol. **66:**5484–5487.

17. **Jorgensen, J. H., and M. J. Ferraro.** 1998. Antimicrobial susceptibility testing: general principles and contemporary practices. Clin. Infect. Dis. **26:**973–980.
18. **Katzif, S., D. Danavall, S. Bowers, J. T. Balthazar, and W. M. Shafer.** 2003. The major cold shock gene, *cspA*, is involved in the susceptibility of *Staphylococcus aureus* to an antimicrobial peptide of human cathepsin G. Infect. Immun. **71:**4304–4312.
19. **Kowalski, R. P., K. A. Yates, E. G. Ramanowski, L. M. Karenchak, F. S. Mah, and J. Gordon.** 2005. An ophthalmologist's guide to understanding antibiotic susceptibility and minimum inhibitory concentration data. Am. Acad. Ophthalmol. **112:**1987–1991.
20. **Kremer, L., Y. Guerardel, S. S. Gurcha, C. Locht, and G. S. Besra.** 2002. Temperature-induced changes in the cell-wall components of *Mycobacterium thermoresistibile*. Microbiology **148:**3145–3154.
21. **Livermore, D. M.** 2003. Bacterial resistance: origins, epidemiology, and impact. Clin. Infect. Dis. **36:**S11–S23.
22. **Lou, Y., and A. E. Yousef.** 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. **63:**1252–1255.
23. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes Acra and Acrb encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
24. **Maciá, M. D., D. Blanquer, B. Togores, J. Sauleda, J. L. Pérez, and A. Oliver.** 2005. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. Antimicrob. Agents Chemother. **49:**3382–3386.
25. **Martinez, J. L., and F. Baquero.** 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. **44:**1771–1777.
26. **McDowell, D. A.** 2004. Food processing stresses in the spread of antibiotic resistance, p. 243–265. *In* F. J. M. Smulders and J. D. Collins (ed.), Safety assurance during food processing: food safety assurance and veterinary public health, vol. 2. Wageningen Academic Publishers, Wageningen, The Netherlands.
27. **Nichols, D. S., J. Olley, H. Garda, R. R. Brenner, and T. A. McMeekin.** 2000. Effect of temperature and salinity stress on growth and lipid composition of *Shewanella gelidimarina*. Appl. Environ. Microbiol. **66:**2422–2429.
28. **Phadtare, S., K. Yamanaka, and M. Inouye.** 2000. The cold shock response, p. 33–45. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, DC.
29. **RahmatiBahram, A., J. T. Magee, and S. K. Jackson.** 1997. Effect of temperature on aminoglycoside binding sites in *Stenotrophomonas maltophilia*. J. Antimicrob. Chemother. **39:**19–24.
30. **Rickard, A. H., S. Lindsay, G. B. Lockwood, and P. Gilbert.** 2004. Induction of the mar operon by miscellaneous groceries. J. Appl. Microbiol. **97:**1063–1068.
31. **Rowan, N. J.** 1999. Evidence that inimical food-preservation barriers alter microbial resistance, cell morphology and virulence. Trends Food Sci. Technol. **10:**261–270.
32. **Santos, J. M., P. Freire, M. Vicente, and C. M. Arraiano.** 1999. The stationary-phase morphogene bolA from *Escherichia coli* is induced by stress during early stages of growth. Mol. Microbiol. **32:**789–798.
33. **Seoane, A., E. Sánchez, and J. M. Garcia-Lobo.** 2003. Tandem amplification of a 28-kilobase region from the *Yersinia enterocolitica* chromosome containing the *blaA* gene. Antimicrob. Agents Chemother. **47:**682–688.
34. **Storz, G., and R. Hengge-Aronis.** 2000. Bacterial stress responses. ASM Press, Washington, DC.
35. **Teuber, M., and J. Bader.** 1976. Action of polymixin B on bacterial membranes. Binding capacities for polymixin B on inner and outer membranes isolated from *Salmonella typhimurium* G30. Arch. Microbiol. **109:**51–58.
36. **Velkov, V. V.** 1999. How environmental factors regulate mutagenesis and gene transfer in microorganisms. J. Biosci. **24:**529–559.
37. **Vieira, H. L. A., P. Freire, and C. M. Arraiano.** 2004. Effect of *Escherichia coli* morphogene *bolA* on biofilms. Appl. Environ. Microbiol. **70:**5682–5684.