

# Exposure of *Escherichia coli* ATCC 12806 to Sublethal Concentrations of Food-Grade Biocides Influences Its Ability To Form Biofilm, Resistance to Antimicrobials, and Ultrastructure

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*Escherichia coli* ATCC 12806 was exposed to increasing subinhibitory concentrations of three biocides widely used in food industry facilities: trisodium phosphate (TSP), sodium nitrite (SNI), and sodium hypochlorite (SHY). The cultures exhibited an acquired tolerance to biocides (especially to SNI and SHY) after exposure to such compounds. *E. coli* produced biofilms (as observed by confocal laser scanning microscopy) on polystyrene microtiter plates. Previous adaptation to SNI or SHY enhanced the formation of biofilms (with an increase in biovolume and surface coverage) both in the absence and in the presence (MIC/2) of such compounds. TSP reduced the ability of *E. coli* to produce biofilms. The concentration of suspended cells in the culture broth in contact with the polystyrene surfaces did not influence the biofilm structure. The increase in cell surface hydrophobicity (assessed by a test of microbial adhesion to solvents) after contact with SNI or SHY appeared to be associated with a strong capacity to form biofilms. Cultures exposed to biocides displayed a stable reduced susceptibility to a range of antibiotics (mainly aminoglycosides, cephalosporins, and quinolones) compared with cultures that were not exposed. SNI caused the greatest increase in resistances (14 antibiotics [48.3% of the total tested]) compared with TSP (1 antibiotic [3.4%]) and SHY (3 antibiotics [10.3%]). Adaptation to SHY involved changes in cell morphology (as observed by scanning electron microscopy) and ultrastructure (as observed by transmission electron microscopy) which allowed this bacterium to persist in the presence of severe SHY challenges. The findings of the present study suggest that the use of biocides at subinhibitory concentrations could represent a public health risk.

*Escherichia coli* strains are common contaminants in the food industry, especially in foods of animal origin (1). This bacterium is part of the common microbiota of the gastrointestinal tracts of animals and human beings, and although most isolates are nonpathogenic and are considered merely indicators of poor hygiene and sanitation conditions (fecal contamination), some 10% to 15% of *E. coli* strains are opportunistic and pathogenic serotypes capable of causing food-borne disease (2, 3). This bacterium is also of concern in the health care system, where it is a frequent nosocomial pathogenic microorganism (4). *Escherichia coli* and *Enterococcus* spp. are considered indicators of antibiotic resistance (5).

Biofilm formation is a major cause for concern in the food system, resulting in serious operational and maintenance costs (decreasing heat transfer and operational efficacy in heat exchange equipment, causing filter plugging and blockages of tubes in water distribution systems, increasing energy consumption, and accelerating damage to surfaces) (6). Moreover, biofilms provide a reservoir of microorganisms, increasing the risk of contamination in food processing plants and leading to critical problems in terms of public health and financial losses (7). Food-borne outbreaks of pathogens associated with biofilms have been related to the presence of *Salmonella*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Staphylococcus aureus*, and *E. coli* (8). Moreover, biofilm formation is considered an important virulence factor in human infections, and it has been reported that approximately 80% of all bacterial infections are associated with biofilms (9, 10).

It is a well-established fact that the metabolic changes and protective effects of biofilm matrices create a conducive environment

for biofilm inhabitants to adapt to higher concentrations of antimicrobial compounds (11). However, there are hardly any reports discussing the reverse phenomenon, i.e., the effect of antimicrobial adaptation on the potential of bacteria to form biofilms. Such a study would provide useful information from a food safety as well as clinical point of view if such adaptations confer a survival advantage upon microorganisms.

Antibiotic resistance is a global public health threat that involves all major microbial pathogens and antimicrobial drugs (12). Recent scientific evidence suggests that the selective pressure exerted by the use of biocides at sublethal concentrations could contribute to the expression and dissemination of antibiotic resistance mechanisms (5). In view of the large and increasing use of biocides, the risk of inappropriate use of such compounds, leading to the selection and spread of antibiotic-resistant bacteria, is of increasing concern, and the need for new research on this topic has been highlighted (5, 13).

An increase in biocide tolerance is also a major public health issue, as it could be expected to contribute to the increased persistence of pathogenic and spoilage bacteria in the food chain. It has been suggested that exposure to sublethal concentrations of biocides could potentially have an impact on the responses of bacteria

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to commonly used food processes, enabling microorganisms to survive challenges such as the concentrations of biocides currently permitted for use in food environments (14). However, at present, there is a limited understanding of the mechanisms which contribute to biocide tolerance.

This study aimed to examine whether exposure of *E. coli* ATCC 12806 to subinhibitory concentrations of three food-grade biocides (trisodium phosphate [TSP], sodium nitrite [SNI], and sodium hypochlorite [SHY]), and consequent adaptation, could influence its ability to form biofilms, resistance to antibiotics, and capacity to survive subsequent severe challenge by such compounds. In addition, the roles of associated changes in the growth kinetics, surface hydrophobicity, morphology, and ultrastructure of *E. coli* cells were examined.

## MATERIALS AND METHODS

***E. coli* strain and culture conditions.** *Escherichia coli* ATCC 12806 was maintained in tryptone soy broth (TSB; Oxoid Ltd., Hampshire, United Kingdom) supplemented with 20% (vol/vol) glycerol at  $-30^{\circ}\text{C}$ . Prior to experiments, frozen cells were subcultured twice in TSB at  $37^{\circ}\text{C}$ . Working cultures were kept at  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$  on plates of tryptone soy agar (TSA; Oxoid) and were subcultured monthly.

**Biocides.** Three compounds were tested: TSP (Merck, Darmstadt, Germany), SNI (Sigma-Aldrich, Steinheim, Germany), and SHY (Sigma-Aldrich). Sterile solutions were prepared in distilled water immediately before each experiment.

**Determination of MICs.** The MIC values were established using a microdilution broth method in accordance with CLSI guidelines (15). Five colonies of *E. coli* were taken from TSA plates, inoculated into 10 ml of Mueller-Hinton (MH) broth (Oxoid), and incubated at  $37^{\circ}\text{C}$ . Previous experiments showed that after 24 h of incubation, these bacterial cultures contained approximately  $10^8$  CFU/ml. For the experiment, 100-well polystyrene microwell plates (Oy Growth Curves Ab Ltd., Helsinki, Finland) were used. Wells were filled with 20  $\mu\text{l}$  of chemical solution (a range of concentrations was used for each biocide) and 180  $\mu\text{l}$  of an appropriate dilution (in MH broth) of inoculum in order to give a final concentration in the well of  $5 \times 10^5$  CFU/ml. The inoculum concentration was confirmed by plating. The microwell plates were incubated at  $37^{\circ}\text{C}$  in a Bioscreen C MBR incubator (Oy Growth Curves Ab). Both positive (200  $\mu\text{l}$  of inoculum at  $5 \times 10^5$  CFU/ml) and negative (180  $\mu\text{l}$  of MH broth plus 20  $\mu\text{l}$  of chemical solution) controls were included in each experiment. The experiments were replicated five times on separate days.

The MIC was established as the lowest biocide concentration necessary to prevent growth after 48 h of incubation. A calibration equation was performed in order to establish a cutoff for bacterial growth. The growth of the strain in MH broth was determined in the following two ways for this purpose: by measuring the optical density at 420 nm to 580 nm ( $\text{OD}_{420-580}$ ) in a Bioscreen C MBR instrument and by enumerating viable cells on plate count agar (PCA; Oxoid) in duplicate, using 0.1% (wt/vol) peptone water (PW; Oxoid) for decimal dilutions, and counting visible colonies after 48 h of incubation at  $37^{\circ}\text{C}$ . Blank sample wells with uninoculated broth were included as a control for contamination. Five replications were carried out on different days. The degree of correlation between optical densities and microbial counts was investigated by linear regression of continuous data.

**Adaptation to increasing concentrations of biocides.** The test was performed in the same manner as that described for determining the MIC. The starting concentration of biocide was MIC/2. When growth was observed, 20  $\mu\text{l}$  of the suspension were aseptically transferred to the next well, which contained 160  $\mu\text{l}$  of MH broth and 20  $\mu\text{l}$  of the chemical solution. After the transfer, each well contained a concentration of biocide 1.5 times stronger than that in the previous well. This procedure was continued until no growth was observed after 72 h of incubation at  $37^{\circ}\text{C}$ . The suspension in the last well with recorded growth was streaked on TSA

plates with biocides (one-half the maximum concentration of biocide that supported microbial growth was added to TSA). Nonexposed cells were grown in TSB and subsequently streaked on TSA plates without biocides. After incubation at  $37^{\circ}\text{C}$  for 48 h, agar plates were kept at  $4^{\circ}\text{C} \pm 1^{\circ}\text{C}$  for no longer than 1 week. For the experiments described below, TSB with biocides (a concentration equivalent to MIC/2 was added) was used for growth of previously adapted cells and TSB without biocides was used in the case of nonadapted cells. Culture broths were inoculated from the cultures kept on TSA (nonadapted cells) or TSA with biocides (adapted cells). All groups of cells (nonadapted and adapted to TSP, SNI, or SHY) were tested simultaneously on the same day of storage on agar plates.

**Biofilm determination.** The study of the formation and structure of biofilms was performed using a previously described method (16), with minimal modifications. Cultures were grown at  $37^{\circ}\text{C}$  for 18 h, and appropriate dilutions in TSB were prepared to obtain a concentration of approximately  $10^6$  CFU/ml. TSB with biocides (MIC/2) was used for growth and dilution of previously adapted cells, and TSB without biocides was used in the case of nonadapted cells. A volume of 250  $\mu\text{l}$  was added to the wells of sterile Matrix 96-well polystyrene flat-bottom microplates (Thermo Scientific, NH) having high optical quality, a low fluorescence background, and overall flatness, which allowed for high-resolution imaging. After 1 h of adhesion at  $37^{\circ}\text{C}$ , the wells were rinsed with 150 mM NaCl in order to eliminate any nonadherent bacteria before being refilled with 250  $\mu\text{l}$  of culture broth. TSB with biocides (MIC/2) and without biocides was added to all groups of wells (containing cells that were nonadapted, adapted to TSP, adapted to SNI, or adapted to SHY). Thus, a total of 16 different conditions were tested. The plate was then incubated for 24 h at  $37^{\circ}\text{C}$ . After the development of biofilms, the cells were rinsed with 150 mM NaCl and refilled with TSB containing 5  $\mu\text{M}$  Syto9 (1:1,000 dilution from a Syto9 stock solution at 5 mM in dimethyl sulfoxide [DMSO]; Invitrogen, Barcelona, Spain), a cell-permeating green fluorescent marker of the nucleic acids of cells. The plate was then incubated in the dark at  $30^{\circ}\text{C}$  for 20 min to enable fluorescence labeling of the bacteria.

Confocal laser scanning microscopy (CLSM) image acquisition was performed using a Nikon Eclipse TE 2000-U confocal laser scanning microscope with EZ-C13.60 software (Nikon Instruments Inc., NY). All biofilms were scanned at 400 Hz, using a  $40\times$  objective lens with a 488-nm argon laser set at 90% intensity. Emitted fluorescence was recorded within the range of 500 nm to 600 nm in order to visualize Syto9 fluorescence. Three stacks of horizontal plane images (512 by 512 pixels, corresponding to 119 by 119  $\mu\text{m}$ ) with a z step of 1  $\mu\text{m}$  were acquired for each biofilm, using different areas in the well. Three independent experiments were performed for each condition, on different days.

For image analysis, three-dimensional projections of the structures of the biofilms were reconstructed using the Easy3D function of IMARIS 7.5 software (Bitplane, Zurich, Switzerland). The quantitative structural parameters of the biofilms, such as their biovolume, substratum coverage, and roughness, were calculated using the computer program COMSTAT (17, 18). The biovolume represented the overall volume of cells ( $\mu\text{m}^3$ ) in the observation field (14.161  $\mu\text{m}^2$ ) and provided an estimate of the biomass in the biofilm. Substratum coverage (%) reflected the efficiency of substratum colonization by the population of bacteria. Roughness provided a measure of how much the thickness of the biofilm varied and is an indicator of biofilm heterogeneity. The maximum thickness ( $\mu\text{m}$ ) of biofilms was determined directly from the confocal stack images.

**Growth curves.** *E. coli* cells were grown in TSB (nonadapted cells) or TSB with biocides (MIC/2; adapted cells) for 18 h at  $37^{\circ}\text{C}$ . Volumes of 30 ml were centrifuged ( $4,000 \times g$ , 10 min,  $4^{\circ}\text{C}$ ), washed with phosphate-buffered saline (PBS; Oxoid), and resuspended in TSB. The wells of microwell plates (Oy Growth Curves Ab) were filled with 200  $\mu\text{l}$  of inoculum (final concentration,  $10^5$  CFU/ml) or with 180  $\mu\text{l}$  of inoculum and 20  $\mu\text{l}$  of chemical solution at an appropriate concentration in order to give a final concentration of biocide in the well of MIC/2. Bacterial growth was monitored before incubation (at hour 0) and every hour until 120 h had elapsed. Growth was determined by measuring the  $\text{OD}_{420-580}$  in a Bio-

screen C MBR instrument. The microwell plates were shaken for 1 min prior to the measurement of turbidity. The experiment was replicated three times, on separate days.

The model used to fit growth curves to the data obtained was the modified Gompertz equation (19):  $OD_t = A + B \times \exp\{-\exp[2.71828183 \times \mu \times (L - t)/B + 1]\}$ , where  $t$  is the time, in hours, that has elapsed since inoculation,  $OD_t$  is the  $OD_{420-580}$  at time  $t$ ,  $L$  is the lag time (hours),  $\mu$  is the maximum growth rate achieved ( $\Delta OD_{420-580}/h$ ),  $B$  is the increase in  $OD_{420-580}$  between the inoculation and the stationary phase (E), and  $A$  is the upper asymptote curve ( $OD_{420-580}$  in the stationary phase [E])  $- B$ . The  $OD_{420-580}$  value at 24 h ( $OD_{24h}$ ) was obtained for each strain and replication by fitting a sigmoidal curve to the data set, using a Marquardt algorithm that calculated the parameter values which gave the minimum residual sum of squares. The goodness of fit was evaluated using the coefficient of determination ( $R^2$ ). The calibration equation performed was used to transform the  $OD_{420-580}$  at 24 h into plate counts ( $\log_{10}$  CFU/ml).

**Determination of CSH.** Microbial cell surface hydrophobicity (CSH) was determined by the microbial adhesion to solvents (MATS) test based on affinity for nonpolar solvents (20). Xylene was used as the hydrocarbon phase. *E. coli* cells were grown in TSB (nonadapted cells) or TSB with biocides (MIC/2; adapted cells) for 18 h at 37°C. Cells were harvested by centrifugation (4,000 × g, 10 min, 4°C) and washed with sterile PBS. All groups of cells were resuspended in TSB and in TSB with biocides (MIC/2) at an initial concentration of 10<sup>5</sup> CFU/ml. Thus, a total of 16 conditions were tested. After 18 h at 37°C, cells were centrifuged, washed twice with PBS, and resuspended in 150 mM NaCl at a concentration of approximately 10<sup>8</sup> CFU/ml. The cell suspension (2.4 ml) was vortexed with 0.4 ml of the solvent for 60 s and then allowed to stand for 15 min at room temperature, which resulted in the complete separation of the two phases. An aqueous-phase sample (1 ml) was obtained, and the absorbance at 400 nm was determined (Bioscreen C MBR). The percentage of cells present in the solvent was calculated with the following equation: % affinity = 100 × [1 - (A/A<sub>0</sub>)], where A<sub>0</sub> is the absorbance of the original suspension at 400 nm prior to mixing and A is the absorbance of the aqueous phase. Each experiment was performed in triplicate with three independently prepared cultures on separate days. Cell surface hydrophobicity was grouped into three categories: weak (<21%), moderate (21% to 50%), and strong (>50%) affinities for xylene (11).

**Determination of antibiotic susceptibility.** *E. coli* cells were screened for susceptibility, before and after adaptation to the chemicals, to a panel of 29 antibiotics on Mueller-Hinton agar (Oxoid) by a disc diffusion method described by the CLSI (15). Adapted cells were grown in TSB with biocides (MIC/2), and nonadapted cells were grown in TSB without biocides. The following antibiotic discs (Oxoid) were used: spectinomycin (SH; 100 µg), amikacin (AK; 30 µg), gentamicin (CN; 10 µg), kanamycin (K; 30 µg), streptomycin (S; 10 µg), tobramycin (TOB; 10 µg), rifampin (RD; 5 µg), ampicillin (AMP; 10 µg), ticarcillin (TIC; 75 µg), amoxicillin-clavulanic acid (AMC; 30 µg), ampicillin-sulbactam (SAM; 20 µg), piperacillin-tazobactam (TZP; 110 µg), cephalothin (KF; 30 µg), cefazolin (KZ; 30 µg), cefoxitin (FOX; 30 µg), cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), cefepime (FEP; 30 µg), imipenem (IPM; 10 µg), aztreonam (ATM; 30 µg), sulfonamides (S3; 300 µg), trimethoprim-sulfamethoxazole (SXT; 25 µg), chloramphenicol (C; 30 µg), nalidixic acid (NA; 30 µg), ciprofloxacin (CIP; 5 µg), enrofloxacin (ENR; 5 µg), tetracycline (TE; 30 µg), phosphomycin (FOS; 50 µg), and nitrofurantoin (F; 300 µg). The inhibition zones were measured and scored as sensitive, intermediate, or resistant according to the CLSI guidelines (15). Cultures of *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 with known antimicrobial resistance patterns were used as reference strains for antibiotic disc control. The stability of resistance to therapeutic antimicrobials was determined by measuring susceptibility to antibiotics for both nonadapted and adapted strains after storage (4°C ± 1°C) for 3 months on TSA plates without biocides, with monthly transfers.

**Determination of resistance to high concentrations of biocides.** *E. coli* cells were grown in TSB (nonadapted cells) or TSB with biocides

(MIC/2; adapted cells) for 18 h at 37°C. Volumes of 30 ml were centrifuged (4,000 × g, 10 min, 4°C), washed with PBS, and resuspended in TSB or TSB with biocides (at 1.5 times the maximum concentration of biocide that allowed bacterial growth after adaptation) at an initial concentration of 10<sup>7</sup> CFU/ml. Initial bacterial concentrations were confirmed by plating. Experimental samples were incubated at 37°C for 60 min. After incubation, 0.75 ml of 100 mM sodium thiosulfate (Na<sub>2</sub>SO<sub>3</sub>; Sigma-Aldrich Química, Madrid, Spain) was added to 30 ml of bacterial suspension to arrest the activity of the biocides prior to sampling.

To determine cell survival, each sample was serially diluted with sterile 0.1% (wt/vol) PW, using 10-fold serial dilutions. After dilution, 0.1-ml samples were taken and spread plated on PCA. Two plates per dilution were prepared, inverted, and incubated at 37°C for 24 h. Each experiment was repeated three times, on separate days. The average number of colonies from the duplicate plates was recorded for each sample. Results are expressed as percentages of CFU after exposure to biocides relative to the initial (preexposure) CFU (percentage of survivors).

**Determination of bacterial morphology and ultrastructure.** *E. coli* cells were tested before adaptation to SHY (nonadapted cells were inoculated in TSB and incubated at 37°C for 18 h) and after adaptation to SHY (cells adapted to SHY were incubated for 18 h at 37°C in TSB with SHY at MIC/2). Both groups of cells were examined before and after exposure to SHY (cells were incubated at 37°C for 60 min in TSB with 1.5 times the maximum concentration of SHY that allowed bacterial growth after adaptation). Thus, a total of four conditions were tested. The cells were harvested by centrifugation (4,000 × g, 10 min, 4°C) and were washed twice by suspension and centrifugation in PBS. *E. coli* cells were fixed in PBS with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., Berkshire, United Kingdom) for 1 h at 4°C. The bacteria were then centrifuged (4,000 × g, 10 min, 4°C) and washed by suspension and centrifugation in PBS. This washing step was repeated twice. For transmission electron microscopy (TEM), cells were treated with 1% osmium tetroxide (TAAB Laboratories Equipment) in PBS for 45 min at room temperature in darkness, followed by three new washing steps. Cells were pelleted in bacteriological agar (Oxoid), and the pellets were thereafter dehydrated in ethanol solutions of increasing concentrations and embedded in an epoxy resin (Epon 812; Tousimis Research Corp., Rockville, MD), which was polymerized by incubation for 48 h at 60°C. Ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate. Observations were made using a JEM 1010 (JEOL Ltd., Tokyo, Japan) microscope at 80 kV. Samples for scanning electron microscopy (SEM) were dried under vacuum, mounted on aluminum stubs, and sputter coated with gold. Samples were viewed with a JSM 6480LV (JEOL) scanning electron microscope operating at 20 kV. Electron microscope observations were documented through the acquisition of representative microphotographs.

**Statistical analysis.** The quantitative structural parameters of biofilms, percentages of affinity for xylene, and bacterial concentrations ( $OD_{420-580}$  or  $\log_{10}$  CFU/ml) in culture broth were compared for statistical significance by using analysis of variance techniques. Mean separations were obtained using Duncan's multiple-range test. The percentages of cultures with increased resistance to antibiotics after exposure to biocides were compared by means of Fisher's exact test. All data processing in this study was carried out using the Statistica 6.0 software package (Statsoft Ltd., Chicago, IL). Significance was determined at the 5% ( $P < 0.05$ ) level.

## RESULTS

**Adaptation of *E. coli* to biocides.** Optical densities ( $OD_{420-580}$ ) gave a linear relationship to microbial counts in the range of 0.200 to 0.700 (7.4 to 8.3  $\log_{10}$  CFU/ml). An  $R^2$  value of 0.842 was obtained over this range. Thus, an  $OD_{420-580}$  of 0.200 was considered the cutoff for bacterial growth in order to calculate the MICs. The regression equation for the relationship between microbial counts



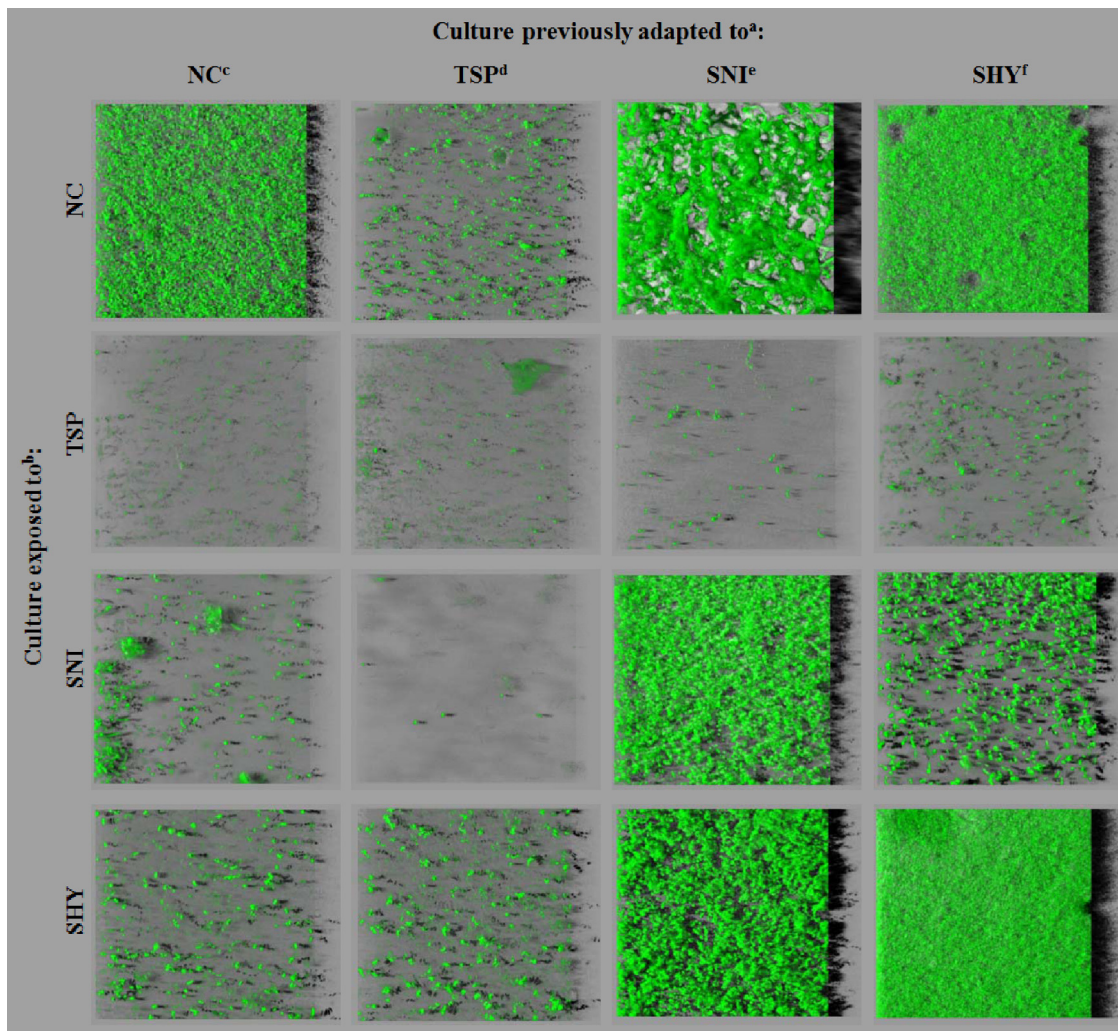


FIG 1 Three-dimensional projections of biofilm structures of *E. coli* ATCC 12806 under 16 different conditions, with shadow projections on the right, obtained from confocal z stacks by use of IMARIS software. a, for adaptation, cultures were previously exposed to increasing subinhibitory concentrations of biocides; b, exposed cultures were grown in the presence of biocides (MIC/2); c, no compound; d, trisodium phosphate; e, sodium nitrite; f, sodium hypochlorite.

( $\log_{10}$  CFU/ml) and  $OD_{420-580}$  values was as follows:  $\log_{10}$  CFU/ml =  $7.061 + (1.809 \times OD_{420-580})$ .

The MICs of TSP, SNI, and SHY for *Escherichia coli* prior to its exposure to subinhibitory concentrations of biocides were 10.428, 14.876, and 0.239 mg/ml, respectively. After several passages through gradually higher concentrations of the compounds, the maximum concentrations of biocides that allowed bacterial growth were 11.731 mg/ml (TSP), 37.655 mg/ml (SNI), and 0.403 mg/ml (SHY). Adaptive tolerance to SNI and SHY was stable after repeated subculture in nonselective broth without biocides (the strain was passed through biocide-free TSB every 24 h for 7 days). On the other hand, in the absence of selective pressure, cells that had adapted to TSP returned to their preadaptation sensitivity.

**Biofilm formation.** *E. coli* cells formed biofilms on polystyrene surfaces under the experimental conditions tested. To determine whether previous exposure to subinhibitory concentrations of biocide (TSP, SNI, or SHY), and consequent adaptation, would influence biofilm production, the architecture of biofilms produced by adapted and nonadapted *E. coli* cells in the absence or

presence of sublethal concentrations (MIC/2) of such compounds was studied.

Representative 24-h biofilm structures observed using CLSM for the cells under study are presented in Fig. 1. The images correspond to three-dimensional reconstructions obtained from confocal stack images by use of IMARIS 7.5. software, including virtual shadow projections on the right. A marked variability in the three-dimensional biofilm architecture was noted between conditions. When cells were grown in the absence of biocides, cells adapted to TSP formed biofilms containing several small aggregates. Nonadapted cells and those adapted to SHY produced rough biofilms with a patchy coverage and confluent growth areas where the bacteria formed clumps. Cells adapted to SNI produced a specific spatial arrangement, forming snake-shaped structures of variable thickness. In the presence of TSP, *E. coli* cells formed only a few, small scattered cell clusters. In the presence of SNI or SHY, small scattered cell clusters were also observed for nonadapted cultures and for cells adapted to TSP, while cells adapted to SNI or SHY displayed a marked ability to form biofilms under these ex-

**TABLE 1** Biovolume values observed for *E. coli* ATCC 12806 adapted and/or exposed to subinhibitory concentrations of food-grade biocides

Culture exposed to <sup>b</sup> :	Biovolume ( $\mu\text{m}^3$ ) of culture previously adapted to <sup>a</sup> :			
	NC	TSP	SNI	SHY
NC	23,664.6 $\pm$ 2,703.9Aa	3,174.1 $\pm$ 613.4Ba	80,467.2 $\pm$ 4,593.5Ca	39,434.1 $\pm$ 1,393.6Da
TSP	509.9 $\pm$ 275.4Ab	857.9 $\pm$ 148.5ABb	435.3 $\pm$ 50.5Ab	1,485.6 $\pm$ 822.3Bb
SNI	2,363.3 $\pm$ 445.6Ab	227.4 $\pm$ 328.7Ab	42,183.0 $\pm$ 3,980.1Bc	6,114.7 $\pm$ 5,480.7Ab
SHY	2,164.0 $\pm$ 296.7Ab	2,798.7 $\pm$ 755.4Aa	45,419.8 $\pm$ 7,564.5Bc	74,474.1 $\pm$ 7,307.5Cc

<sup>a</sup> For adaptation, cultures were previously exposed to increasing subinhibitory concentrations of biocides. Data are means  $\pm$  SD for nine determinations. Means in the same row with no capital letters in common are significantly different ( $P < 0.05$ ). Means in the same column with no lowercase letters in common are significantly different ( $P < 0.05$ ). NC, no compound; TSP, trisodium phosphate; SNI, sodium nitrite; SHY, sodium hypochlorite.

<sup>b</sup> Cultures were grown in the presence of biocides (at MIC/2).

perimental conditions. It should be noted that cells adapted to SHY produced compact structures that covered most of the surface available in the presence of this compound.

Biovolume, substratum coverage, maximum thickness, and roughness parameters were extracted from confocal stack images in order to quantify biofilm structures with numerical data that would allow statistical analysis (Tables 1 to 4). Numerical data confirmed the visual observations. In TSB without biocides, cells adapted to TSP produced the smallest biofilms, with low biovolume (biomass) and surface coverage figures. Cultures adapted to SNI or SHY formed stronger biofilms (with a higher percentage of covered surface and larger biovolume) than did nonadapted strains. In the presence of TSP, *E. coli* cells formed weak biofilms, irrespective of adaptation. In the presence of SNI and SHY, strains adapted to these compounds produced biofilms with a larger biovolume and more surface coverage than those of nonadapted strains or those adapted to TSP.

Strong correlations ( $P < 0.001$ ) were observed between biovolume and substratum coverage ( $r = 0.906$ ), between biovolume and maximum thickness ( $r = 0.634$ ), and between substratum coverage and maximum thickness ( $r = 0.471$ ). No significant correlations ( $P > 0.05$ ) were observed between roughness and the remaining structural parameters of biofilms (biovolume, surface coverage, and maximum thickness), thus suggesting similar levels of heterogeneity of biofilm thickness under the different conditions tested.

A three-dimensional scatterplot was drawn up to show the relationship between biovolume, substratum coverage, and roughness (Fig. 2). As can be seen, cells may be classified into two main groups (conditions 1, 9, 11 to 13, and 16 versus conditions 2 to 8, 10, 14, and 15).

The possible influences of growth kinetics (concentration of bacteria in the culture broth in contact with the polystyrene surface at 24 h of incubation, the time at which biofilm formation was determined) and of CSH on biofilm formation were tested. In-

cluding the replicates, a total of 48 OD<sub>420–580</sub> curves were generated for the *E. coli* cells tested and fitted to the modified Gompertz equation. The  $R^2$  values for the Gompertz model fit were high ( $>0.90$ ). The OD<sub>420–580</sub> values at 24 h (OD<sub>24h</sub>) were extracted from these curves. The regression equation obtained between microbial counts ( $\log_{10}$  CFU/ml) and OD<sub>420–580</sub> values ( $\log_{10}$  CFU/ml = 7.061 + 1.809  $\times$  OD<sub>420–580</sub>) was used to estimate microbial counts after 24 h of incubation. Thus, OD<sub>24h</sub> values obtained throughout the experiment were transformed into microbial counts in accordance with this equation. The estimated OD<sub>24h</sub> values and microbial counts ( $\log_{10}$  CFU/ml) recorded after 24 h are shown in Table 5. In the absence of biocides, similar cell densities were found in adapted and parent (not previously adapted) cultures. In TSB with TSP, SNI, or SHY, the highest cell density under each condition was found for cells adapted to TSP, SNI, or SHY, respectively.

Important differences in hydrophobicity values (ranging from 4.5  $\pm$  0.3 to 66.2  $\pm$  2.4) were found between cells, depending on the conditions tested (Fig. 3). Weak ( $<20\%$  affinity for xylene), moderate (21% to 50% affinity), and strong ( $>50\%$  affinity) reactions were observed for 6 (37.5%), 4 (25.0%), and 6 (37.5%) conditions, respectively. The highest values for cell surface hydrophobicity were shown by cells adapted and/or exposed to SNI, followed by those adapted and/or exposed to SHY. The lowest figures were observed for cultures adapted and/or exposed to TSP.

The relationship between surface coverage of the biofilm (%), estimated concentration of bacteria in TSB at 24 h ( $\log_{10}$  CFU/ml), and hydrophobicity (percentage of adhesion to xylene) was examined. In the case of cells growing in the absence of biocides, similar concentrations of bacteria in TSB were observed for the four conditions tested (adapted and nonadapted cells) after 24 h of incubation at 37°C (Table 5). No substantial differences were found between the hydrophobicity values of parent cells and cells previously adapted to either TSP or SHY (Fig. 3). However, cul-

**TABLE 2** Surface coverage values observed for *E. coli* ATCC 12806 adapted and/or exposed to subinhibitory concentrations of food-grade biocides<sup>a</sup>

Culture exposed to:	Surface coverage (%) of culture previously adapted to:			
	NC	TSP	SNI	SHY
NC	74.0 $\pm$ 3.7Aa	14.0 $\pm$ 2.2Ba	80.2 $\pm$ 1.88Ca	85.1 $\pm$ 3.1Ca
TSP	3.1 $\pm$ 1.5Ab	4.5 $\pm$ 0.5ABb	1.1 $\pm$ 0.2Ab	7.3 $\pm$ 3.5Bb
SNI	9.9 $\pm$ 1.7Ac	0.4 $\pm$ 0.5Bc	80.4 $\pm$ 5.3Ca	26.8 $\pm$ 1.8Dc
SHY	8.7 $\pm$ 0.1Ac	10.6 $\pm$ 3.3Aa	80.9 $\pm$ 2.1Ba	91.2 $\pm$ 8.0Ca

<sup>a</sup> For information on interpretation, see the footnotes to Table 1.

**TABLE 3** Maximum thicknesses observed for *E. coli* ATCC 12806 adapted and/or exposed to subinhibitory concentrations of food-grade biocides<sup>a</sup>

Culture exposed to:	Maximum thickness ( $\mu\text{m}$ ) of biofilms of culture previously adapted to:			
	NC	TSP	SNI	SHY
NC	19.7 $\pm$ 0.6ABa	17.3 $\pm$ 1.5Aa	36.0 $\pm$ 2.0Ca	20.3 $\pm$ 0.6Bab
TSP	21.0 $\pm$ 2.7Aa	21.3 $\pm$ 1.5Aa	14.3 $\pm$ 3.2Bb	21.0 $\pm$ 1.0Aab
SNI	20.7 $\pm$ 4.0Aa	8.0 $\pm$ 1.0Bb	20.0 $\pm$ 1.0Ac	18.3 $\pm$ 2.3Aa
SHY	23.0 $\pm$ 1.0Aa	19.0 $\pm$ 1.0Ba	23.0 $\pm$ 2.0Ac	23.3 $\pm$ 2.1Ab

<sup>a</sup> For information on interpretation, see the footnotes to Table 1.

TABLE 4 Roughness values observed for *E. coli* ATCC 12806 adapted and/or exposed to subinhibitory concentrations of food-grade biocides<sup>a</sup>

Culture exposed to:	Roughness value of culture previously adapted to:			
	NC	TSP	SNI	SHY
NC	0.47 ± 0.01Aa	0.47 ± 0.02Aab	0.53 ± 0.14Aa	0.46 ± 0.01Aab
TSP	0.35 ± 0.07Ab	0.39 ± 0.06Aa	0.49 ± 0.07Aa	0.39 ± 0.09Aa
SNI	0.48 ± 0.02Aa	0.61 ± 0.14Ab	0.47 ± 0.01Aa	0.52 ± 0.02Ab
SHY	0.51 ± 0.03Aa	0.55 ± 0.09Aab	0.49 ± 0.02Aa	0.40 ± 0.04Ba

<sup>a</sup> For information on interpretation, see the footnotes to Table 1.

tures adapted to TSP showed the least ability to form biofilms (low surface coverage) (Table 2). Cells adapted to SNI showed both the highest hydrophobicity values and the greatest potential for forming biofilms.

In the presence of TSP, minimal figures were observed for both hydrophobicity and surface coverage, even though moderate concentrations of bacteria in the culture broth were found. When cells were grown in the presence of SNI, a high hydrophobicity and a low bacterial concentration were observed for both nonadapted and adapted cells. The highest surface coverage of the biofilm was found for cells adapted to SNI, followed by cells adapted to SHY. Nonadapted cells and those adapted to TSP showed a low surface coverage. In the presence of SHY, no substantial differences were found between conditions with regard to the bacterial concentration at 24 h. However, cells adapted to SNI and SHY showed the highest values for surface coverage and hydrophobicity.

**Antibiotic susceptibility.** *E. coli* cultures were screened for susceptibility to 29 antibiotics before and after exposure to sublethal concentrations of biocides. Before adaptation, *E. coli* was susceptible to all antibiotics tested by the disc diffusion method. The exposure of *E. coli* to increasing subinhibitory concentrations

of biocides was associated with reductions in the susceptibility to several antibiotics (Table 6).

The number of increased resistances caused by SNI (14 [48.28% of the antibiotics tested]) was larger ( $P < 0.05$ ) than those for TSP (1 [3.44%]) and SHY (3 [10.34%]). The impact of biocide exposure on reduced susceptibility to antibiotics depended on the antibiotic family tested, with aminoglycosides (especially spectinomycin), cephalosporins, and quinolones (especially nalidixic acid) showing the greatest increases in resistance. The reduced susceptibility to antibiotics as a result of adaptation to biocides remained stable after 3 months in the absence of biocides (*E. coli* cells were maintained on TSA plates and subcultured monthly).

**Resistance to strong concentrations of biocides.** *E. coli* cultures (initial concentration of  $10^7$  CFU/ml) were incubated at 37°C for 1 h in biocide-free TSB or in TSB with biocides (in this case, 1.5 times the maximum doses that supported microbial growth after adaptation) prior to plating to determine viability.

No differences between groups of cells were observed in terms of the morphology of the colonies on TSA. In the absence of biocides or in the presence of SNI, the population density after incubation was essentially stable. *E. coli* cells were completely killed by the presence of TSP. Lastly, when *E. coli* cells were exposed to SHY, the bacterial population decreased substantially during the 1-h test period (Table 7).

In the absence of biocides, the bacterial concentrations during the study period were similar for adapted and nonadapted cells. The influence of previous adaptation on bacterial resistance to TSP or SNI was slight. On the other hand, extreme differences in susceptibility of bacteria to inhibition by SHY were found with respect to their previous adaptation. Thus, after exposure to high

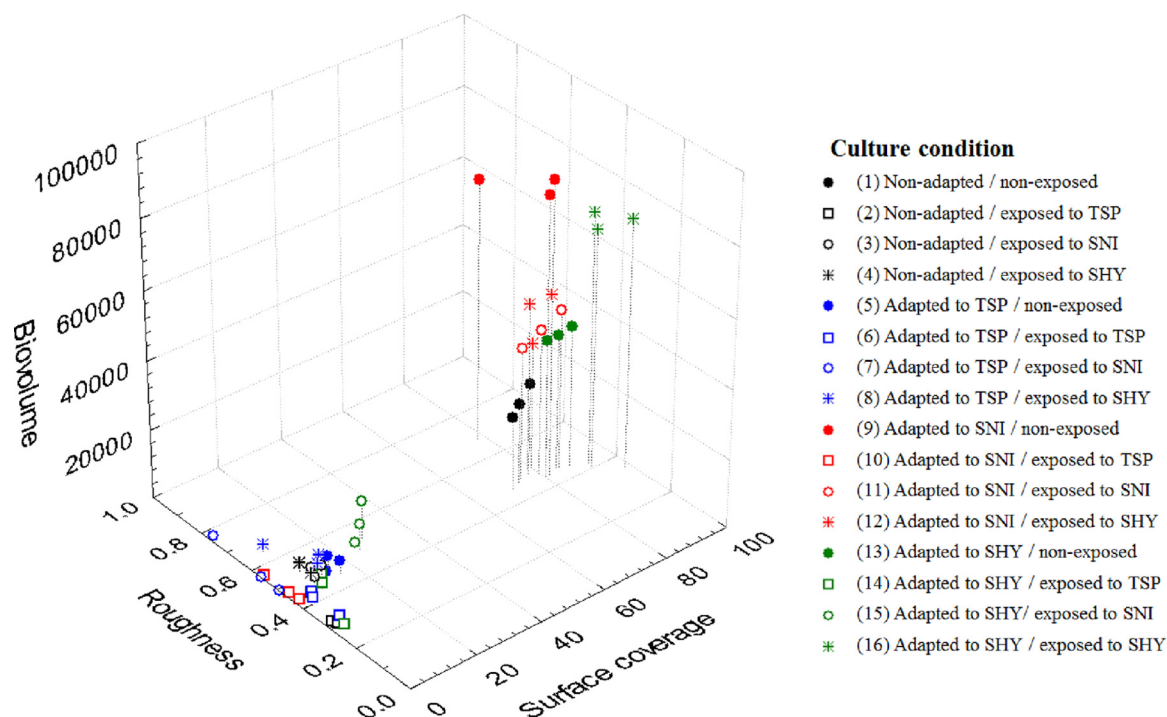


FIG 2 Relationships between biovolume ( $\mu\text{m}^3$ ), surface coverage (%), and roughness of biofilms produced by *E. coli* ATCC 12806 adapted and/or exposed to sublethal concentrations of food-grade biocides. Data are the averages for three determinations. For additional interpretation, see the legend to Fig. 1.



TABLE 5 Optical densities and bacterial concentrations observed for *E. coli* ATCC 12806 in tryptone soy broth after 24 h of incubation at 37°C<sup>a</sup>

Culture exposed to:	OD <sub>24h</sub> (log <sub>10</sub> CFU/ml) of culture previously adapted to:			
	NC	TSP	SNI	SHY
NC	0.68 ± 0.07Aa (8.30 ± 0.13Aa)	0.66 ± 0.04Aa (8.26 ± 0.08Aa)	0.59 ± 0.02Aa (8.14 ± 0.03Aa)	0.61 ± 0.07Aa (8.17 ± 0.12Aa)
TSP	0.44 ± 0.02ABb (7.85 ± 0.03ABb)	0.47 ± 0.05Ab (7.92 ± 0.09Ab)	0.46 ± 0.01ABb (7.89 ± 0.01ABb)	0.40 ± 0.04Bb (7.78 ± 0.06Bb)
SNI	0.20 ± 0.01Ac (7.42 ± 0.01Ac)	0.24 ± 0.04ABc (7.49 ± 0.08ABc)	0.28 ± 0.05Bc (7.56 ± 0.09Bc)	0.20 ± 0.01Ac (7.41 ± 0.02Ac)
SHY	0.30 ± 0.01Ad (7.60 ± 0.02Ad)	0.31 ± 0.02Ac (7.62 ± 0.04Ac)	0.26 ± 0.00Bc (7.53 ± 0.01Bc)	0.43 ± 0.04Cb (7.84 ± 0.07Cb)

<sup>a</sup> OD<sub>24h</sub>, OD<sub>420–580</sub> after 24 h. OD<sub>420–580</sub> values were transformed to log<sub>10</sub> CFU/ml according to the regression equation obtained [log<sub>10</sub> CFU/ml = 7.061 + (1.809 × OD<sub>420–580</sub>)]. Data are means ± SD for three determinations. For additional information on interpretation, see the footnotes to Table 1.

concentrations of SHY for 1 h, cells adapted to SHY showed a >250-fold higher percentage of survivors than nonadapted cells or those adapted to TSP or SNI. Thus, the morphological and ultrastructural changes in *E. coli* cells associated with adaptation and/or exposure to SHY were investigated in order to determine the possible mechanisms of acquired resistance to this compound.

**Determination of bacterial morphology and ultrastructure in the presence of SHY.** To elucidate why bacterial adaptation to SHY decreases the deleterious effect of high concentrations of this compound, morphological and ultrastructural changes in *E. coli* occurring after adaptation and/or exposure to SHY were examined by SEM and TEM.

The SEM analysis (Fig. 4) revealed that after adaptation to SHY, ridges appeared on the outer surfaces of the cells. Moreover, the cells appeared thinner and longer than control (nonadapted) cells. After exposure to high concentrations of SHY, the adapted cultures displayed few or no morphological changes relative to nonexposed cultures. However, after challenge by SHY, nonadapted cultures exhibited considerable morphological modifications in comparison with control (nonexposed) cultures. Modifications ranged from deformed cells, with individual bumps, buds, grooves, and cavities, to overall cell surface wrinkling. Cell wall disruption was observed in the form of cracks, holes, or even cell lysis. In some places, clusters of numerous lysed and fused cells were seen.

Transmission electron microscopy examination of cells adapted to SHY confirmed the changes (elongation) in the nature

of the cellular suspension compared with nonadapted cultures (Fig. 5). In addition, *E. coli* adapted to SHY showed electron-dense regions and evidence of cytoplasmic condensation. In *E. coli* cells exposed to high concentrations of SHY (both adapted and nonadapted cells), the cytoplasm was disorganized and the inner and outer membranes were separated. No uniform spacing between the *E. coli* cytoplasmic contents and the cell envelope was observed. Exposure to SHY caused major structural changes in nonadapted cells. Thus, in contrast to adapted bacteria after exposure to SHY, nonadapted *E. coli* cells showed coagulated material, especially in the periphery of the cells, and a very wide space between the cytoplasm and the cell membranes. Cultures exhibited wavy cell envelope structures, with the presence of blebs in the outer membrane, and the integrity of the outer membrane was not maintained in some cases, demonstrating that the *E. coli* cells were lysed after exposure to SHY.

## DISCUSSION

**Adaptation of *E. coli* to biocides.** *E. coli* ATCC 12806 exhibited acquired tolerance to biocides (especially SNI and SHY) after exposure to increasing subinhibitory concentrations of such compounds. The increased tolerance to SNI or SHY remained after 7 successive passages in biocide-free TSB, suggesting that in *E. coli* ATCC 12806, adaptive tolerance to both compounds could be the result of heritable mutations. These findings are in agreement with reports by other authors, where adaptation of *E. coli* to both chemical and physical sublethal stresses has been demonstrated (21).

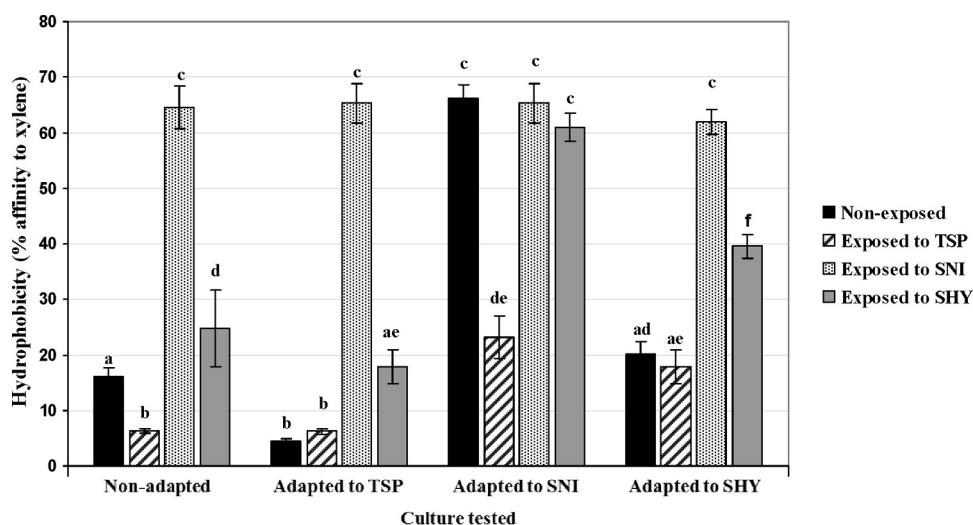


FIG 3 Hydrophobicity values observed for cultures of *E. coli* ATCC 12806 tested under 16 different conditions. Data are means ± standard deviations (SD) for three determinations. Mean values with no letters in common are significantly different ( $P < 0.05$ ). For additional interpretation, see the legend to Fig. 1.

TABLE 6 Antibiotic resistance patterns of *E. coli* cultures tested

<i>E. coli</i> cultures previously adapted <sup>b</sup> to:	Resistance to indicated antibiotic <sup>a</sup>																												
	SH	AK	CN	K	S	TOB	RD	AMP	TIC	AMC	SAM	TZP	KF	KZ	FOX	CTX	CAZ	FEP	IPM	ATM	S3	SXT	C	NA	CIP	ENR	TE	FOS	F
NC	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TSP	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SNI	R	R	S	R	R	I	S	S	S	S	S	S	R	R	S	R	R	R	S	R	I	S	S	R	I	R	S	R	R
SHY	R	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S

<sup>a</sup> SH, spectinomycin (100 µg); AK, amikacin (30 µg); CN, gentamicin (10 µg); K, kanamycin (30 µg); S, streptomycin (10 µg); TOB, tobramycin (10 µg); RD, rifampin (5 µg); AMP, ampicillin (10 µg); TIC, ticarcillin (75 µg); AMC, amoxicillin-clavulanic acid (30 µg); SAM, ampicillin-sulbactam (20 µg); TZP, piperacillin-tazobactam (110 µg); KF, cephalothin (30 µg); KZ, cefazolin (30 µg); FOX, cefoxitin (30 µg); CTX, cefotaxime (30 µg); CAZ, ceftazidime (30 µg); FEP, cefepime (30 µg); IPM, imipenem (10 µg); ATM, aztreonam (30 µg); S3, sulfonamides (300 µg); SXT, trimethoprim-sulfamethoxazole (25 µg); C, chloramphenicol (30 µg); NA, nalidixic acid (30 µg); CIP, ciprofloxacin (5 µg); ENR, enrofloxacin (5 µg); TE, tetracycline (30 µg); FOS, fosfomycin (50 µg); F, nitrofurantoin (300 µg). Resistances are indicated as follows: R, resistant strain; I, strain of intermediate susceptibility; and S, susceptible strain. Data for exposed strains with increased resistance to antibiotics (relative to unexposed strains) are shaded; values for exposed strains not exhibiting increased susceptibility to antibiotics are not shaded. An increase in resistance was defined as a change from S (before exposure) to R (after exposure).

<sup>b</sup> For adaptation, cultures were previously exposed to increasing subinhibitory concentrations of biocides. For additional information on interpretation, see the footnotes to Table 1.

The increased tolerance observed suggests that the use in food environments of compounds which may provide sublethal exposure when used inappropriately (improper use, inappropriate storage, or the presence of excessive amounts of organic matter, known to inactivate several biocides) represents a real risk for the development of adaptation to biocides. Caution should also be exercised when food preservation treatments are used at low intensities. It is a frequent practice worldwide to use combined preservative factors (hurdles) at sublethal doses because of their synergistic antimicrobial effect. Hurdle technology aims to improve the microbial status and the sensory quality of foods, as well as their nutritional properties (22). The potential link between preservation treatments at low intensities and microbial acquisition of new or modified characteristics, such as more resistance to antimicrobials, has been suggested previously (12, 23).

**Biofilm formation.** The ability of *E. coli* to attach to, colonize, and form biofilms on different surfaces is well documented (24). However, only limited studies have thus far been conducted to determine the influences of adaptation and/or exposure to sublethal doses of biocides on the potential to form biofilms, and it appears that the effect of contact with TSP, SNI, or SHY on the ability of *E. coli* to form biofilms may have been tested for the first time in this work.

*E. coli* cells that had neither been adapted nor exposed to biocides produced rough biofilms on polystyrene, with clusters and irregular coverage, as previously described both under static conditions (16) and in continuous-flow systems (25). These findings are a matter for concern, because plastic materials are used extensively in surfaces and equipment throughout the food chain (10). Moreover, several studies have shown a correlation between bio-

film production on polystyrene microwells and biofilm formation on the surfaces of different materials in food facilities (26, 27).

The finding of substantially improved biofilm formation ability after adaptation to SNI or SHY reported here, which is in agreement with similar observations in earlier studies performed with different bacterial species and chemical compounds (1, 28–30), suggests that the use of antimicrobial compounds at inappropriate doses in the food industry may increase the ability of bacteria to produce biofilms, thus imposing a food safety threat because the presence of biofilms on food processing surfaces favors the contamination of foodstuffs.

The concentration of bacteria in the culture broth in contact with the polystyrene surface at 24 h of incubation (biofilm formation was evaluated at this time point), as well as the cell surface hydrophobicity of *E. coli* cultures, was studied in order to improve our understanding of the differences in the ability to form biofilms between cultures subjected to different conditions. The cell densities of the culture broths were found not to have any apparent relationship to their biofilm-producing capability. Thus, for TSB with and without compounds, important differences in biofilm formation ability were observed between groups of cells (non-adapted or adapted to TSP, SNI, or SHY), even though no substantial differences in bacterial concentrations were found. These results agree with findings from other authors, who found that the number of attached cells remained constant over the period of incubation (31, 32). It has been suggested that after a few hours of incubation, the surface reaches a saturation level where larger numbers of planktonic cells do not entail larger numbers of attached cells (7). This is one possible explanation for the absence of any relationship between biofilm formation and the density of

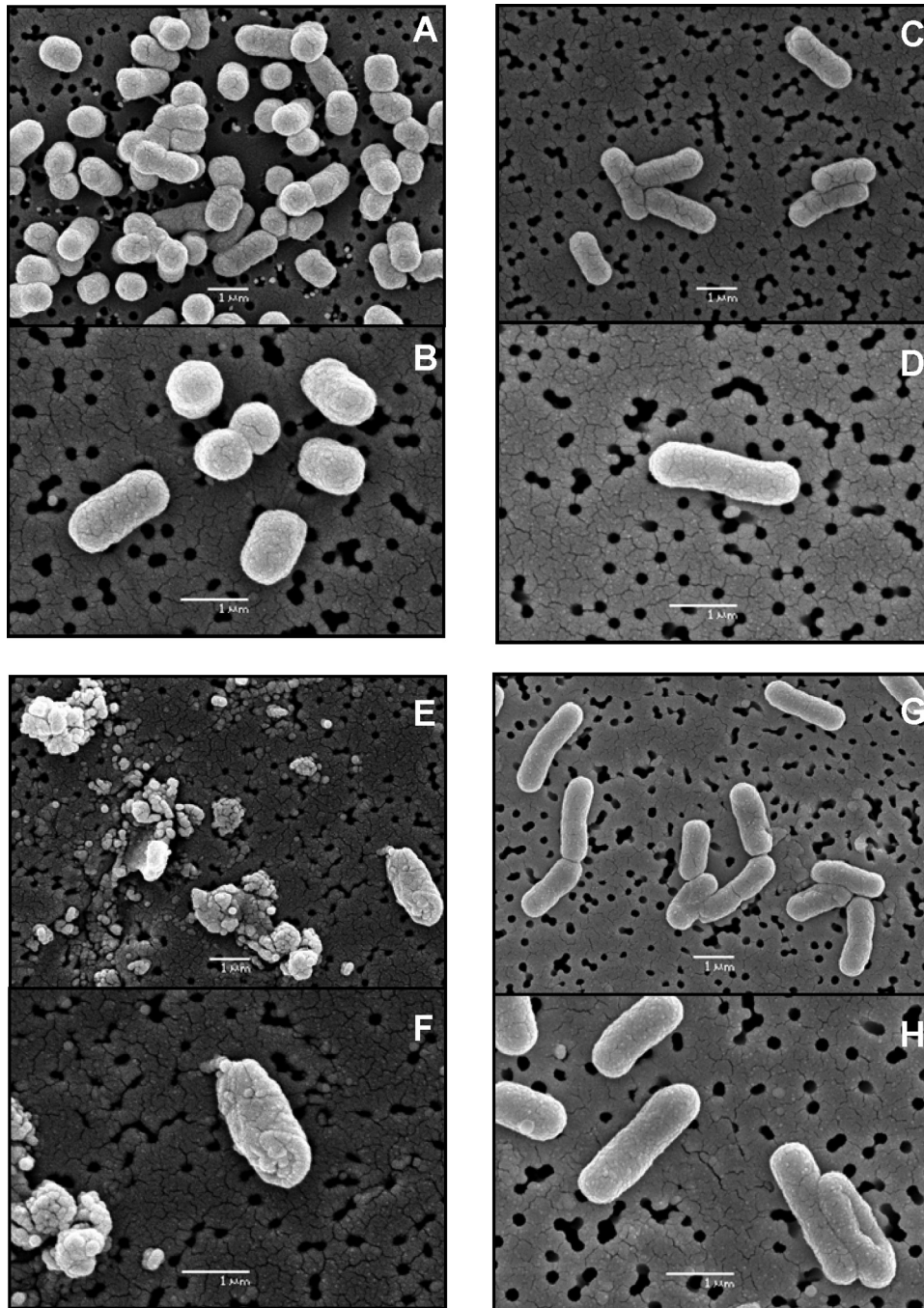
TABLE 7 Survival of *E. coli* ATCC 12806 after incubation for 1 h at 37°C in TSB or TSB with biocides

Culture incubated in the presence of <sup>b</sup> :	% survival of culture previously adapted to <sup>a</sup> :			
	NC	TSP	SNI	SHY
NC	133.87 ± 35.07Aa	103.89 ± 57.19Aa	99.94 ± 40.40Aa	108.44 ± 26.84Aa
TSP	0.00 ± 0.00Ab	0.00 ± 0.00Ab	0.00 ± 0.00Bb	0.00 ± 0.00Bb
SNI	118.35 ± 30.95ABa	111.47 ± 48.72ABa	101.73 ± 34.15Aa	145.95 ± 27.67Bc
SHY	0.08 ± 0.08Ab	0.22 ± 0.15Ab	0.00 ± 0.00Ab	56.20 ± 25.56Bd

<sup>a</sup> Values represent the concentrations of bacteria after incubation with regard to initial concentrations (%). For adaptation, cultures were previously exposed to increasing subinhibitory concentrations of biocides. Data are means ± SD for three determinations. For additional information on interpretation, see the footnotes to Table 1.

<sup>b</sup> Cultures were incubated at 37°C for 60 min in TSB with 1.5 times the maximum concentration of biocide that allowed bacterial growth after adaptation.



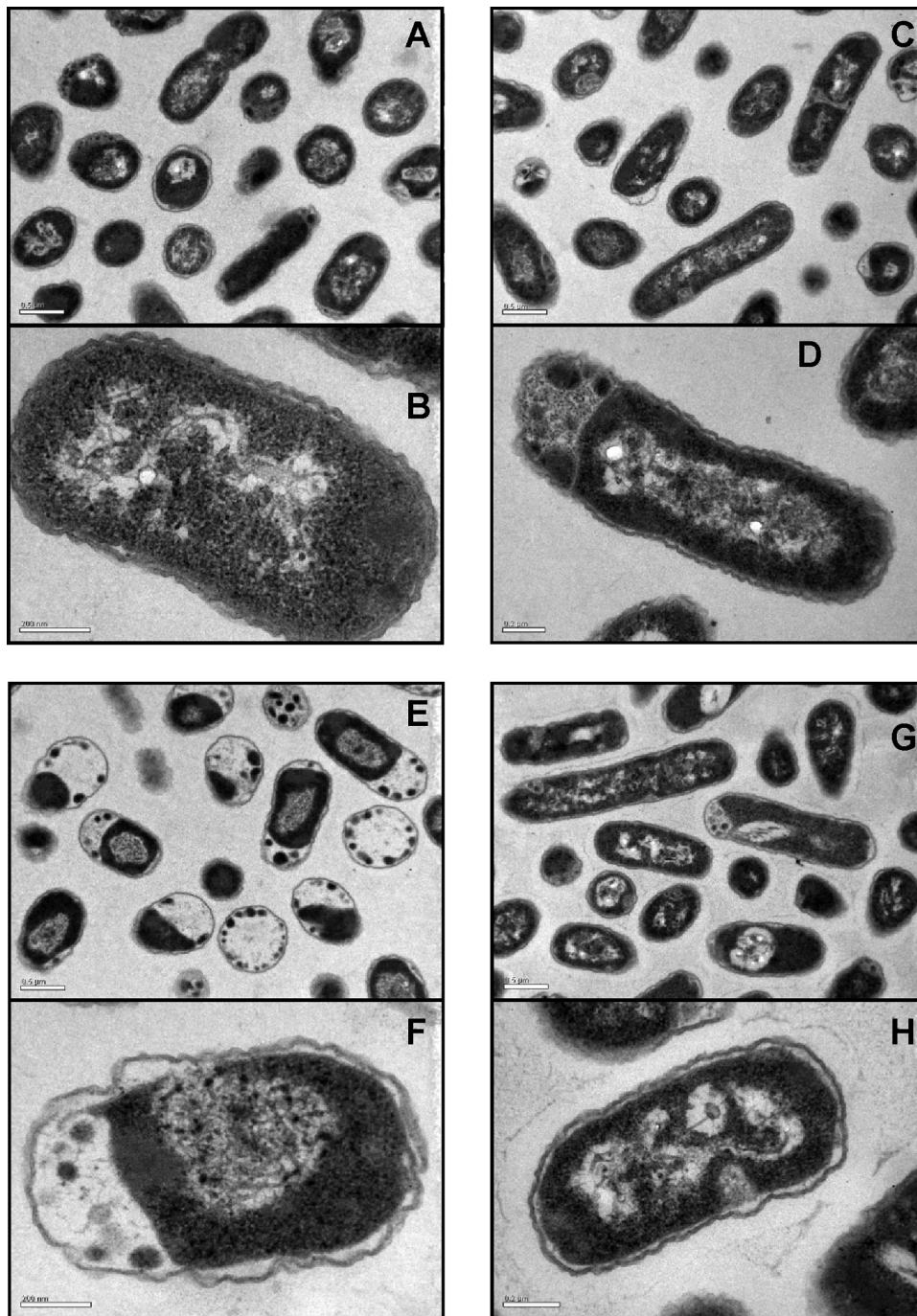


**FIG 4** Scanning electron micrograph sections of *E. coli* ATCC 12806 cells. (A and B) Nonadapted untreated cells; (C and D) adapted untreated cells; (E and F) nonadapted treated cells; (G and H) adapted treated cells. For adaptation, cultures were previously exposed to increasing subinhibitory concentrations of sodium hypochlorite. Treated cells were exposed to sodium hypochlorite at 1.5 times the maximum concentration of biocide that allowed bacterial growth after adaptation.

suspended cells after 24 h of incubation in the present study. It must be pointed out, however, that these results should be considered with caution, because the different sizes of *E. coli* cells under different growth conditions (Fig. 4 and 5) could bring into question the relationship between cell density (determined in this study) and microbial counts in some circumstances.

As an adaptive response, bacterial cell surfaces may undergo

chemical modifications to repel water-soluble compounds. These modifications may result in a change in cell surface hydrophobicity. The hydrophobicity of the microbial cell surface plays a critical role in the adherence of bacteria to a wide variety of hydrophobic surfaces (e.g., polystyrene) (33, 34). Because adherence of bacteria to the surface is the first step in biofilm formation, it is expected that an increase in cell surface hydrophobicity is accompanied by



**FIG 5** Transmission electron micrograph sections of *E. coli* ATCC 12806 cells. (A and B) Nonadapted untreated cells; (C and D) adapted untreated cells; (E and F) nonadapted treated cells; (G and H) adapted treated cells. For adaptation, cultures were previously exposed to increasing subinhibitory concentrations of sodium hypochlorite. Treated cells were exposed to sodium hypochlorite at 1.5 times the maximum concentration of biocide that allowed bacterial growth after adaptation.

an increase in bacterial ability to form biofilms on polystyrene surfaces (11, 26, 35). However, controversial results on the effect of cell surface hydrophobicity on biofilm formation on cell contact surfaces have been reported (24).

The hydrophobicity values observed for parent *E. coli* cells grown in the absence of biocides ( $16.1 \pm 1.6$ ) are in agreement with figures reported by other authors for different *E. coli* serovars,

which showed that *E. coli* strains exhibit low hydrophobicity (4). However, comparisons between reports should be considered with caution because several factors (e.g., characteristics of the culture medium, growth conditions, variability of the bacterial strains, presence of antimicrobials, or methods used in the study) may modify the hydrophobic surface properties of bacterial cells (26, 36).



Parent (nonadapted) *E. coli* cells growing in the absence of biocides showed a weak hydrophobicity ( $16.1 \pm 1.6$ ), a high estimated bacterial concentration ( $8.30 \pm 0.13 \log_{10}$  CFU/ml), and a considerable surface coverage by the biofilm ( $74.0\% \pm 3.7\%$ ). Adaptation and/or exposure to biocides significantly modified these figures (Tables 2 and 5; Fig. 3).

The increase in cell surface hydrophobicity observed after contact (adaptation and/or exposure) with sublethal concentrations of SHY and, especially, SNI could be related to a reduction in the charged (hydrophilic) binding sites for the biocides (37). Changes in hydrophobicity do not explain the differences observed in the ability to form biofilms in some cases. Thus, similar levels of surface coverage were observed in TSB without biocides for cells adapted to SNI (which showed high values for hydrophobicity) and for those adapted to SHY or nonadapted (with lower hydrophobicity figures), thus suggesting that biofilm formation in *E. coli* ATCC 12806 is regulated by different factors.

The cell surface hydrophobicity of *E. coli* cells increased in the presence of SNI, with figures in the same range being obtained for both nonadapted and adapted strains (Fig. 3). Despite these similar values (figures of a like nature were also observed for parent and adapted cultures in the case of cell density), cells adapted to SHY and SNI had a greater ability to produce biofilms than nonadapted cells and those adapted to TSP. Thus, factors other than hydrophobicity and cell density might influence the biofilm-forming potential of *E. coli* in the presence of SNI. Along these lines, several reports have shown that bacterial adhesion to surfaces is the first step in biofilm formation. Once adhered to a surface, bacteria aggregate to form microcolonies organized in the three-dimensional structure of a biofilm. However, high levels of initial adherence (associated with high cell surface hydrophobicity) do not necessarily lead to the formation of thick biofilms (38). On the other hand, the hydrophobicity values seen had a strong influence on biofilm formation in the presence of SHY, as cells with the highest hydrophobicity values showed the greatest potential to form biofilms (Table 2; Fig. 3).

Lastly, cells adapted and/or exposed to subinhibitory concentrations of TSP showed little potential to form biofilms, together with low values for hydrophobicity, highlighting the positive effects of TSP in the context of food safety. Several authors (39, 40) have suggested that changes in cell surface properties with alkaline and/or other environmentally induced damage can significantly disrupt cell metabolism and structure, preventing effective interactions between bacterial cells and their environment. This fact might explain the limited biofilm formation ability of cells adapted and/or exposed to TSP. In addition to the high pH and ionic strength of the TSP solutions, the ability of TSP to remove fat films and to have a surfactant or detergent effect (41) may be responsible for the limited ability of *E. coli* to form biofilms after contact with this compound.

**Antibiotic susceptibility.** Concerns about a possible linkage between adaptation to biocides and antibiotic resistance have been growing for a number of years and have been raised at both the national and international levels (12, 42). If pathogenic bacteria are resistant to commonly used antibiotics, then this causes an added problem for people acquiring infections. It is noteworthy that the study presented here revealed an increase in resistance to quinolones after adaptation to biocides. Fluoroquinolones are critically important drugs for treating serious *E. coli* infections in humans, and continued surveillance is required to detect emerg-

ing fluoroquinolone-resistant phenotypes (43). The movement of strains from the category of “sensitive” to that of “resistant” observed for aminoglycosides and cephalosporins after exposure to subinhibitory concentrations of biocides (marked for aminoglycosides) is also a matter for concern in view of the fact that these compounds are the front-line antimicrobials for treating serious bacterial infections in humans (44, 45). It should be pointed out, however, that clinical levels of resistance were not reached and that data indicating public health relevance are lacking.

SNI caused the largest number of increased resistances (14), followed by SHY (3). Adaptation to TSP caused only one increased resistance (to AMP). These results are not surprising because among the compounds tested, SNI showed the largest increases in the maximum concentration of biocides that allowed bacterial growth after adaptation. According to Braoudaki and Hinton (46), a relationship between insensitivity to biocides and resistance to antibiotics is generally seen. The decreases simultaneously observed in susceptibility to SNI and antibiotics, which have different modes of action, suggest a nonspecific resistance mechanism. It has been suggested that the linkage between biocide adaptation and resistance to antibiotics in Gram-negative bacteria might be due to nonspecific efflux pumps. In fact, the classes of antibiotics showing a marked increase in resistance after exposure to biocides (aminoglycosides, cephalosporins, and quinolones) are known efflux pump substrates (47). Changes in the cell surface which induce a reduction in cell permeability do not allow chemically unrelated molecules into the resistant cells and also play a vital role in the establishment of resistance to a variety of antimicrobial agents (48). In the present study, cultures with high cell surface hydrophobicity (adapted to SNI) also showed an increased resistance to antibiotics. Similarly, Braoudaki and Hinton (46) reported that increased cell surface hydrophobicity was associated with antibiotic resistance in *Salmonella* strains.

**Resistance to strong concentrations of biocides.** This work demonstrated that *E. coli* ATCC 12806 has the capacity to resist inactivation by SHY after exposure to sublethal concentrations of such a compound. This finding is of particular concern because SHY is commonly used in food processing facilities and other environments to reduce or eliminate pathogenic and spoilage microorganisms. Even though SHY is generally used at concentrations above those employed in this research, suboptimal concentrations of biocide could occur as a consequence of improper use (erroneous concentration or inadequate distribution), inappropriate storage of the formulations (resulting in a decrease in the effective biocide concentration), or the presence of excessive amounts of organic matter (known to inactivate several chlorinated compounds) (5).

An increase in *E. coli* tolerance to biocides after exposure to sublethal concentrations has been demonstrated widely for triclosan, benzalkonium chloride, and chlorhexidine (14, 49). In order to avoid an increase in biocide tolerance, several biocidal agents (with various modes of action on their microbial target organisms) should be combined in the development of commercial biocides (50). Moreover, the consequences of adaptation can be limited by alternating the formulations used over time (14).

**Determination of bacterial morphology and ultrastructure in the presence of SHY.** One major way in which microorganisms cope with their environment is to alter their morphology, and various morphological adaptations have been reported and reviewed (51). The elongation observed after adaptation, probably



due to disturbances in cell division, is in agreement with previous findings on tolerance to environmental stresses (20, 39, 52). Moreover, the changes in the surfaces of adapted cells, which were more undulating and rougher than those of parent cells, suggest the involvement of modifications of the outer membrane of *E. coli* in its tolerance to SHY. Outer membrane remodeling has been shown to be partially responsible for the adaptive resistance of Gram-negative bacteria to several antimicrobials (53, 54).

The electron-dense regions observed in TEM examinations of *E. coli* cells exposed to SHY are likely due to the accumulation of glycogen inclusion bodies (55). Other authors (56, 57) reported similar electron-dense granules in TEM examinations following *E. coli* exposure to low concentrations of biocides (silver ions and mineral leachates, respectively). Coagulated cytoplasmic material was observed near the cell wall of treated *E. coli*. This coagulated material is thought to be a precipitate of abnormal proteins or denatured membrane (58, 59). The treated cells also showed numerous breaks in the cell membranes. Similar observations have been reported as evidence of outer membrane damage (60, 61). Notably, these ultrastructural alterations were not evident in the previously adapted cells, thus indicating that adaptation to SHY increases bacterial resistance to high concentrations of this compound.

A characteristic effect of SHY on the ultrastructure of *E. coli* cells seems to be the formation of blebs on the outer membrane of the cell envelope. These findings are consistent with corresponding studies of Gram-negative bacteria, in which it was also found that bacteria treated with antimicrobials present these structures (62). As in the study presented here, these reports described thin-section projections that appeared to consist of a unit of membrane clearly continuous with the whole outer membrane, without participation of the entire cell envelope. The physical cause for these changes in outer membrane morphology may be due to the loss of certain outer membrane proteins and/or lipopolysaccharides (63).

The findings of the present study have improved our understanding of the mechanism of SHY's antibacterial action. Upon exposure to SHY, membrane integrity is compromised and structural integrity is not retained. We suggest that the morphological and structural changes induced by sublethal doses of SHY are among the possible mechanisms of resistance of *E. coli* to high concentrations of this compound.

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