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Original article

Selection and electrophoretic characterization of *Salmonella enterica* subsp. *enterica* biocide variants resistant to antibiotics

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Abstract

The proposed research outlines a serious common concern of *Salmonella* resistance to antimicrobials following prolonged exposure to the disinfectants (biocides). These phenotypes of bacteria could potentially result in hard to treat infections. Typical for avian sources, biocide sensitive *S. enterica* subsp. *enterica* serovars: Typhimurium, Enteritidis, Virchow and Zanzibar and their isogenic biocide-tolerant variants were studied in order to investigate bacteriostatic effect of two commercially available biocide formulations: potassium peroxymonosulfate (P) and dodecylamine based structure (triamine, D). We found that cultivating of the bacteria in the medium supplemented with a blend containing P did not influence their antibiotic susceptibility pattern. In contrast, tolerance of bacteria to D compound resulted in resistance to co-trimoxazole, cefotaxime and ciprofloxacin of which two cefotaxime and ciprofloxacin are used commonly for the treatment of invasive *Salmonella* infections in humans. The dependency between OMP patterns and the level of *Salmonella* survival in media containing the biocides was observed merely in serovar Typhimurium. In conclusion, these results suggest that *Salmonella* strains challenged by prolonged treatment with the disinfectants become resistant to antibiotics, however it depends on *Salmonella* serovar and the chemical used. This paper also highlights the loop-mediated isothermal amplification (LAMP) as a technique that offers great benefits to microbiological detecting of *Salmonella* species in the samples.

Key words: *Salmonella*, biocide, antibiotic, resistance, LAMP, outer membrane proteins

Introduction

Salmonella is an important zoonotic pathogen of economic significance in both humans and animals. *Salmonella* infection remains the second most commonly identified gastrointestinal disease across the European Union (EU) (Eurosurveillance 2013, Afema et al. 2014). The reported incidence of

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Table 1. Veterinary industry and healthcare environment biocide formulations used in this study (manufacturers' instructions).

Biocide Formulation no.	Active agent(s)	Recommended contact time (min)	Experimental contact time (see Table 2)	Recommended working concentration	Experimental working concentration	Mechanisms of action
1	potassium peroxymon osulfate, surfactant, organic acids	10	24 days	1 g/100 ml	From 0.13 g/100 ml to 1.5 g/100 ml	Oxidizing sulphur bonds in proteins and enzymes Disrupting the function of the cell membrane causing rupturing of the cell wall
2	triamine, bromine, ethanol, EDTA (tetrasodium salt), Lutensol XL 90, citric acid	1-10	24 days	0.5 ml/100 ml – 5 ml/100 ml	From 0.01 ml/100 ml to 0.18 ml/100ml	Membrane disruption Damaging proteins Loss of cell contents due to lysis

Salmonella infection has been declining steadily since 2004, partly due to EU control programmes in poultry farms. However, *Salmonella* continues to be the source of many epidemic outbreaks due to the spreading of antimicrobial resistant (AMR) strains. The transfer of resistant bacteria from food-producing animals to humans is most evident in human bacterial pathogens originating from food animal sources, such as *Salmonella*, which has reservoirs in cattle, chickens, pigs, and turkeys. For this reason it is important to detect any occurrence of resistance and increases in resistance levels (Angulo et al. 2004).

In connection with the Regulation (EC) No. 2160/2003 of the European Parliament and of the Council on the control of *Salmonella* and other specified food-borne zoonotic agents, and the following Directives, the Home Programme of Eradication of some *Salmonella* serotypes in broilers (species *Gallus gallus*) was introduced in Poland. In the cases of bird salmonellosis, the district veterinary surgeon demands careful cleaning and disinfection of hen houses. These are the first places of bacteria contact with different antimicrobials, to a considerable degree with the disinfectants. The proposed research outlines a serious common concern of bacterial cross-resistance, which means their low susceptibility to chemicals similar in structure or function. Bacteria that survive a low-level dose of biocides are more likely to be resistant to antibiotics (Whitehead et al. 2011, Futoma-Kołoch et al. 2013). The cause of this phenomenon is possible excessive or incorrect usage of biocides and disinfectants leading to the selection of variant strains resistant to the antibiotics (Su et al. 2004, Giraud et al. 2006). Biocides are inorganic, or synthetic organic molecules used to disinfect, sanitize, or sterilize objects and surfaces. There is still a lack of understanding of the mode of action of biocides against pathogens, especially when used at low or subinhibitory concentrations.

Bacteria use the same three major disinfectant resistance strategies employed to achieve resistance to antibiotics: target alteration, inactivation, and reduction in target access (Chapman 2003). Reduction in target access can be accomplished by exclusion or efflux, so the resistance of *Salmonella* strains to antibiotics *sensu lato* followed by biocide resistance manifests in different outer membrane protein (OMPs) patterns (Olliver et al. 2005, Giraud et al. 2006, Karatzas et al. 2008). The data suggest that the relationship between functioning of the efflux pumps can be a common mechanism of bacterial cell survival against biocides. Hence, the analysis of the OMPs can lead to the development of the drugs active against AMR *Salmonella* isolates.

The aim of this paper was to investigate the antibiotic resistance phenomenon in *Salmonella* strains isolated from humans as the result of adaptation to the increasing concentrations of two biocidal formulations containing potassium peroxymonosulfate (P) and dodecylamine based structures (triamine, D) (Table 1). In this paper we employed a novel, rapid, and specific loop-mediated isothermal amplification (LAMP) assay for confirmation of the presence of *Salmonella* strains in the specimens (Futoma-Kołoch et al. 2014).

Materials and Methods

Disinfectants and antibiotics

Disinfectants: biocide formulation with triamine (D) (Amity International) or potassium per-

Table 2. Generation of potassium peroxymonosulfate (P) and dodecylamine (D) tolerant *Salmonella* variants. (+) growth of bacteria in broth supplemented with the biocide seen as the turbidity of the tubes contents in LB broth or the presence of the colonies typical for *Salmonella* bacteria on XLD, (-) lack of growth in medium, nt – not tested, bST – strains transferred to phenotype stability test, aST – after stability test.

		Salmonella enterica subsp. *enterica* serovar			
		Enteritidis	Typhimurium	Virchow	Zanzibar
Time of incubation	MIC	**potassium peroxymonosulfate (P) (mg/ml)**			
7 days in LB broth	subMIC 0.5 x MIC	1.3 +	1.3 +	1.8 +	1.8 +
Gradient 4 x 4 days in LB broth	0.75 x MIC	2.0 +	2.0 +	**2.6^{bST}** +	**2.6^{bST}** +
	1.0 x MIC	2.5 +	2.5 +	3.5 –	3.5 –
	1.25 x MIC	3.0 +	3.0 +	4.4 –	4.4 –
	1.5 x MIC	3.8 +	3.8 +	nt	nt
1 day on XLD agar	2 x MIC	**5.0^{bST}** +	**5.0^{bST}** +	nt	nt
	4 x MIC	10.0 –	10.0 –	nt	nt
	6 x MIC	15.0 –	15.0 –	nt	nt
10 days in LB broth		Stability test			
aST		**3.0** +	**3.0** +	**3.5** +	**3.5** +
Time of incubation	MIC	**dodecylamine (D) (µl/ml)**			
7 days in LB broth	subMIC 0.5 x MIC	0.2 +	0.1 +	0.1 +	0.1 +
Gradient 4 x 4 days in LB broth	0.75 x MIC	0.2 +	0.1 +	0.1 +	0.1 +
	1.0 x MIC	0.3 +	0.2 +	0.1 +	0.1 +
	1.25 x MIC	0.4 +	0.2 +	0.2 +	0.2 +
	1.5 x MIC	0.5 +	0.2 +	0.2 +	0.2 +
1 day on XLD agar	2 x MIC	**0.6^{bST}** +	**0.3^{bST}** +	**0.3^{bST}** +	**0.3^{bST}** +
	4 x MIC	1.2 –	0.6 –	0.5 –	0.5 –
	6 x MIC	1.8 –	0.9 –	0.8 –	0.8 –
10 days in LB broth		Stability test			
aST		**0.4** +	**0.2** +	**0.3** +	**0.3** +

Antibiotics: ciprofloxacin, co-trimoxazole, cefotaxime, amoxicillin/clavulanic acid and ampicillin were purchased from Oxoid.

Bacteria

Four most frequently reported *Salmonella* serovars in avian sources were used in the studies: *Salmonella enterica* subsp. *enterica* serovars: Enteritidis, Typhimurium, Virchow, and Zanzibar collected between 2009-2011 in the Provincial Sanitary-Epidemiological Station in Wrocław.

Isolation of biocide tolerant variants and stability of their phenotypes

Isolation of variants from populations of *Salmonella* was done according to Ricci *et al.* (Ricci et al. 2006) and Karatzas *et al.* (2008) (Table 2). Wild-type strains of *Salmonella* were exposed to: I) subinhibitory concentrations of the disinfectants relevant to 0.5 x MIC for 7 days, II) gradually increasing concentrations of the same substance (4 days for each concentration), III) one-day incubation in LB broth containing 2-fold, 4-fold, and 6-fold increase in biocides MICs, and IV) ten-days incubation in LB broth, in the absence of the disinfectant to test the stabilities of the phenotypes (stability test, STsuperscript in Table 2).

Confirmation of *Salmonella* isolates on XLD and with LAMP technique

Bacterial strains were pre-enriched, grown overnight in Luria-Bertani broth at 37°C under aerobic conditions. DNA isolation was performed with Genomic Mini Kit (A&A Biotechnology) according to the manufacturer's instructions. The LAMP reaction was done with the Ampli-LAMP *Salmonella* species kit (Novazym, Poland) according to Notomi et al. (2000).

Antimicrobial susceptibility

The testing was done using disc diffusion and E-test method. Parent strains and their biocide tolerant variants were tested by the broth microdilution method to determine minimum inhibitory concentration (MIC) of antimicrobials followed by interpretation according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2015) epidemiological cut-off values and clinical breakpoints.

Preparation of bacterial outer membrane proteins (OMP)

The isolation of OMPs from bacteria was performed with ReadyPrep™ Protein Extraction Kit, Membrane I (BioRad). OMPs samples were purified with ProteoExtract™ Protein Precipitation Kit (Calbiochem). Protein quantification in OMP samples was done with a bicinchoninic acid (BCA) Protein Assay Kit (PIERCE®) according to Smith (1985).

Polyacrylamide Gel Electrophoresis (SDS-PAGE) of OMP

OMPs were analysed according to the Laemmli buffer system (Laemmli 1970) using 7.5% stacking gel and 12.5% separating gel. Ten-microliter samples (10 µg/10 µl) were applied. Gel loading was normalized according to bacterial density (OD_{600}=1.0) at the starting point of the preparations. Electrophoresis was conducted at 35 mA of constant current. The OMPs were visualised with Coomassie Brilliant Blue. Results were confirmed in three independent experiments.

Molecular Analyses of OMP

The OMPs were analysed with the Quantity One® 1-D Analysis Software, v. 4.6.3. (Bio-Rad).

Results

Biocide exposure experiment

To investigate if the prolonged exposition of the bacteria to the increasing concentration of the disinfectant influences their ability to growth the experiments in which cultures of *Salmonella* in LB broth supplemented with each tested biocide were used. Data in Table 2 show that *S*. Enteritidis, *S.* Typhimurium, *S.* Virchow and *S*. Zanzibar grown in LB broth supplemented with P or D in the subinhibitory concentrations (0.5 x MIC). In the increasing gradient of P (from 0.75 x MIC to 2 x MIC, that was 2.0 mg/ml to 5.0 mg/ml) the growth of *S*. Typhimurium and *S.* Enteritidis was observed but *S*. Virchow and *S*. Zanzibar were able to grow merely in LB broth containing P in the concentration of 2.6 mg/ml (0.75 x MIC). In contrast, four serovars developed a D tolerance phenotype, with a two-fold increase in the MIC values. Neither P biocide tolerant variants (PV) nor D biocide tolerant variants (DV) were isolated in the arrangements with the disinfectants used in the concentrations of 4 x MIC and 6 x MIC.

Table 3. Susceptibility of parent strains and their biocide variants to antibiotics.

Strain	Antibiotic/chemotherapeutic				
	CIP (5 µg)	SXT (25 µg)	CTX (5 µg)	AMX 30 (20/10 µg)	AMP (10 µg)
Enteritidis – parent strain	S	S	S	S	I
Enteritidis DV bST	S	**R**	**R**	I	I
Enteritidis DV aST	S	**S**	**S**	I	I
Enteritidis PV bST	S	S	S	I	I
Enteritidis PV aST	S	S	S	I	I
Typhimurium – parent strain	S	S	S	I	**R**
Typhimurium DV bST	**R**	**R**	S	I	**I**
Typhimurium DV aST	**S**	**S**	S	I	**I**
Typhimurium PV bST	S	S	S	I	R
Typhimurium PV aST	S	S	S	I	R
Virchow – parent strain	S	S	S	S	I
Virchow DV bST	S	S	S	I	I
Virchow DV aST	S	S	S	I	I
Virchow PV bST	nt	nt	nt	nt	nt
Virchow PV aST	nt	nt	nt	nt	nt
Zanzibar – parent strain	S	S	S	S	**R**
Zanzibar DV bST	S	S	S	I	**I**
Zanzibar DV aST	S	S	S	I	**I**
Zanzibar PV bST	nt	nt	nt	nt	nt
Zanzibar PV aST	nt	nt	nt	nt	nt

PV – potassium peroxymonosulfate variant, DV – dodecylamine variant, bST – before stability test, aST – after stability test, S – sensitive, R – resistant, I – intermediate, nt – not tested, Antibiotics: CIP – ciprofloxacin, SXT – co-trimoxazole, CTX – cefotaxime, AMX 30 -amoxicillin/clavulanic acid, AMP – ampicillin.

oxymonosulfate (P) (DuPont). *Salmonella* variants were tested for MIC determination before and after ST to verify if the feature of biocide resistance is stable or not. As can be seen, the resistance of *S.* Enteritidis and *S*. Typhimurium strains to biocides decreased after the stability test.

Antibiotic susceptibility profiling

We have found that the passage of *Salmonella* in medium containing tested biocidal formulations enabled selection of variants resistant to antibiotics (Table 3). The resistance of the *S.* Enteritidis DV bST to co-trimoxazole and cefotaxime was observed. In the case of *S.* Typhimurium DV bST, resistance to ciprofloxacin and co-trimoxazole was noted. Additionally, the interesting susceptibility tendency was documented for *S.* Typhimurium variants (DV bST, DV aST) and *S.* Zanzibar variants (DV bST, DV aST), which recovered sensitivity to ampicillin in comparison to the parent strains that were resistant to this antibiotic. In the group of PV strains any resistance pattern against antibiotics was observed.

Salmonella confirmation

Prolonged passage of the bacteria during the selection experiments brings the risk of contaminations with other microorganisms. Two parent strains and eight biocide variants were positively tested on the base of typical for *Salmonella* ladder-like pattern of DNA bands (Fig. 1A). Isolates were also identified as *Salmonella* spp. on XLD agar plates as the colonies were red-yellow with black centers (Fig. 1B).

OMP analysis

SDS-PAGE was used to compare the OMP patterns of parent *Salmonella* strains and their biocide variants generated in media supplemented with D or P. Very similar electrophoretic band patterns for the major OMPs of the tested strains of *Salmonella* were obtained. The dependency between OMP patterns and the level of *Salmonella* survival in media containing biocides was observed merely on serovar

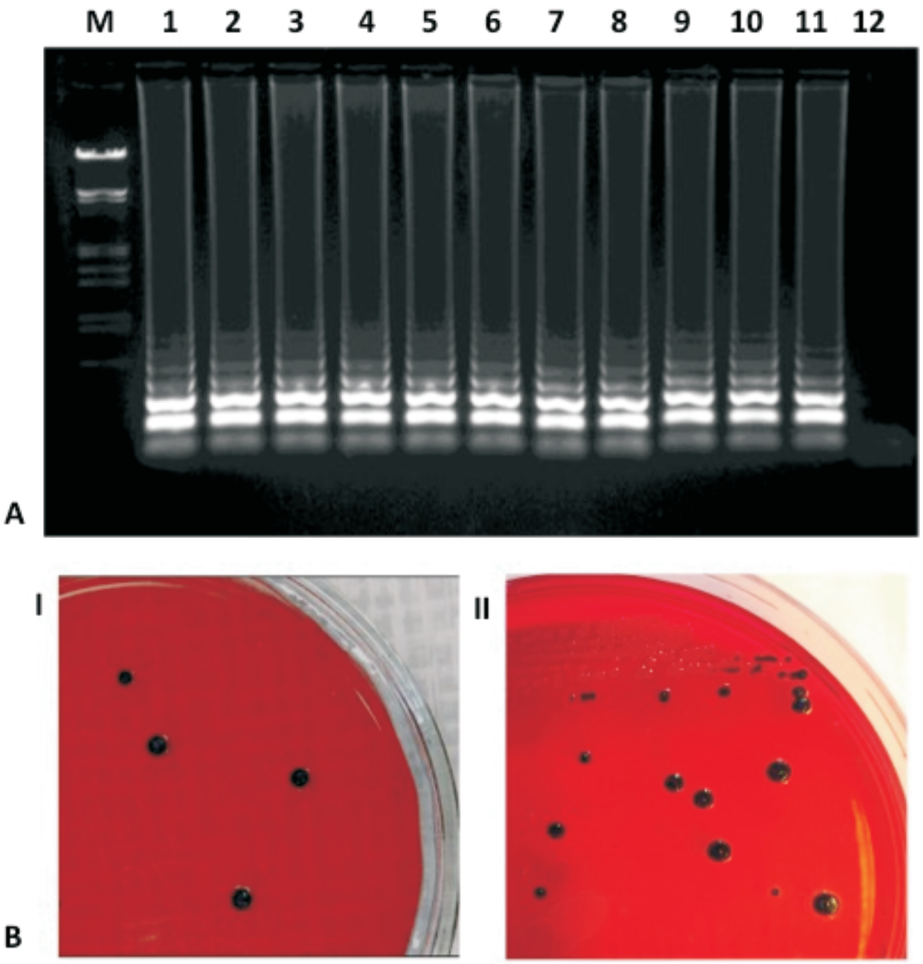

Fig. 1. Confirmation of *Salmonella* strains. **A.** Representative gel image generated with the LAMP technique. M-DNA Marker, Lambda DNA/EcoR1+HindIII Marker, Lane 1 – *S.* Enteritidis ATCC 13076; Lane 2 – *S*. Enteritidis parent strain, Lane 3 – *S.* Typhimurium parent strain; Lane 4 – *S*. Enteritidis PV bST, Lane 5 – *S*. Enteritidis DV aST, Lane 6 – *S.* Typhimurium PV bST, Lane 7 – *S*. Typhimurium DV aST, Lane 8 – *S*. Virchow PV bST, Lane 9 – *S*. Virchow DV aST, Lane 10 – *S*. Zanzibar PV bST, Lane 11 – *S*. Zanzibar DV aST, Lane 12 – control reaction without DNA template. **B. I.** *S.* Typhimurium DV bST (2 x MIC), and **II.** *S.* Typhimurium DV aST isolates identified as *Salmonella spp*. after growing on XLD agar plates. DV -dodecylamine variant, PV – potassium peroxymonosulfate variant, bST – before stability test, aST – after stability test.

Typhimurium (data for the rest strains are not shown) (Fig. 2). In the Lane 2 (parent strain), 55-kDa protein band is readily visible in contrast to the Lanes relevant to *S.* Typhimurium DV bST (Lane 3), DV aST (Lane 4) OMPs in which the band of this molecular mass is less apparent.

Discussion

Disinfectants, which are used widely in farm environments, may select for antibiotic-resistant pathogens. As suggested by Condell et al. (2012), it is possible that tolerance to a disinfectant may arise following incorrect use of the formulation, for example when the biocide is used at concentrations below that which is recommended by the manufacturer or if it becomes diluted accidentally.

There have been relatively few studies that have looked at emerging bacterial resistance due to use of biocides. Stable variants of *Salmonella* obtained following treatment with a quaternary ammonium disinfectant containing formaldehyde and glutaraldehyde, an oxidizing compound blend and a phenolic tar acid-based disinfectant exhibited reduced susceptibility to ciprofloxacin, chloramphenicol, tetracycline, and ampicillin and showed reduced levels of outer membrane proteins (Karatzas et al. 2007, Karatzas et al. 2008). In 2013, we first observed the *S.* Enteritidis and *S*. Typhimurium cross-tolerance to biocides and antibiotics (Futoma-Kołoch et al. 2013). In this paper, we have demonstrated the data that the formulation containing active agents such as triamine can select for co-resistant *S.* Enteritidis, *S*. Typhimurium, S. Virchow, and S. Zanzibar. The two tested biocides were

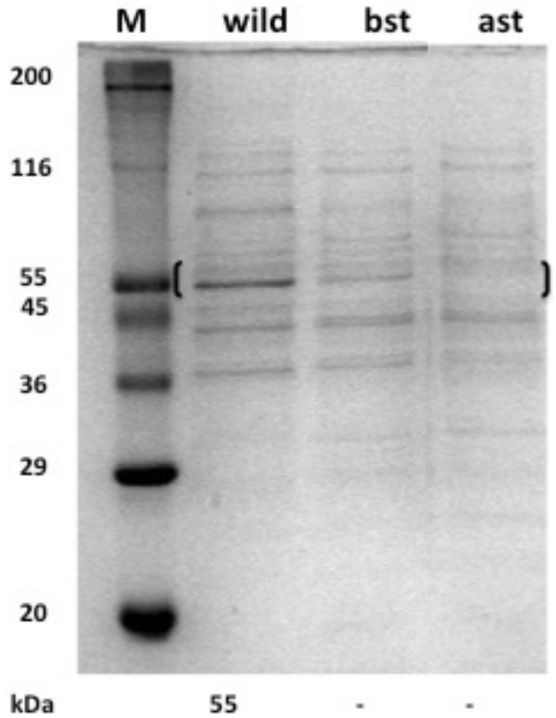

Fig. 2. Representative electrophoregram of SDS-PAGE showing differences in *S*. Typhimurium OMPs patterns. Lane 1, molar mass marker S8445 (Sigma), Lane 2 - *S*. Typhimurium parent strain, Lane 3 – *S*. Typhimurium DV bST (2 x MIC), Lane 4 – *S*. Typhimurium DV aST. DV – dodecylamine variants. bST – before stability test, aST – after stability test.

chosen on the basis of their low price from a vast group of commonly accessible commercial preparations. The resistance to co-trimoxazole and cefotaxime was noted for *S.* Enteritidis and *S*. Typhimurium that demonstrated resistance to ciprofloxacin and co-trimoxazole. On the other hand, the loss of the resistance of parent strains of *S*. Typhimurium and *S*. Zanzibar to ampicillin was also observed.

Challenge of *Salmonella* strains from various origins (such as clinical sources, food, the environment, and water) with commercial biocidal formulations or its components only yielded stable tolerance phenotypes for the single components but not to the biocidal formulations (Condell et al. 2012). Biocidal variants generated in our experiments also have not stable phenotypes probably for the reason of using the formulations, not each component separately.

Surprisingly, these data show that *Salmonella* belonging to the same species *S. enterica* present distinctly susceptible patterns to antibacterials following repeated exposure to disinfectants. Additionally, using the biocide containing P against bacteria, in the concentration of almost ten times lower than that recommended by the manufacturer, did not lead to the selection of antibiotic-resistant mutants. However, antibacterial-resistance pattern was influenced by prolonged treatment of the bacteria with the biocide containing D in the concentration of almost 100 times lower than that recommended by the manufacturer. *S.* Enteritidis D and *S*. Typhimurium D variants became resistant to the antibiotics of the first-line treatments FQs and cephalosporins but only in *S*. Typhimurium co-resistant strain OMPs pattern changes were observed. However, it is worth emphasizing that using of the biocides in the concentrations recommended by the manufactures minimize the risk of Salmonella resistance to the antibacterials.

On the basis of the results obtained from SDS-PAGE analysis we speculate that the resistance of *S.* Typhimurium DV to ciprofloxacin and cefotaxime is possible to be connected to the 55-kDa protein lower expression. Related studies of other authors have shown that exposure of *S.* Typhimurium to four different biocides (an oxidative compound, a QAC, a mixture of aldehydes and a QAC, and a halogenated tertiary amine compound) at their recommended use concentrations selected for multi-drug resistant mutants with a de-repressed AcrEF multidrug efflux pump (Whitehead et al. 2011) or low levels of OmpC and OmpF (Karatzas et al. 2008). It would seem likely that low levels of OMP determine the reduced susceptibility of *Salmonella* to some antibiotics but, presumably, these changes are not sufficient to alter the disinfectant MICs. Systematic and in-depth investigation of biocide-resistant bacteria can provide insight into strategies to subvert biocide resistance, and answer significant questions about the occurrence of antibiotic cross- and co-resistance in *Salmonella*.

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