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Substrate specificity of the OqxAB multidrug resistance pump in *Escherichia coli* and selected enteric bacteria

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***Objectives*: A plasmid-encoded multidrug efflux pump, OqxAB, identified in *Escherichia coli* of porcine origin, was tested for substrate specificity against selected antibiotics, detergents and disinfectants. The ability of horizontal transfer to food-borne pathogens of the Enterobacteriaceae family was also investigated.**

***Methods*: The MICs of selected substrates were determined with a broth dilution assay using two isogenic *E. coli* strains, except for the presence of the *oqxAB* operon. A derivative of the plasmid encoding OqxAB (pOLA52) was constructed and horizontal transfer to *Salmonella* Typhimurium, *Klebsiella pneumoniae*, *Kluyvera* sp. and *Enterobacter aerogenes* was investigated. The effect of the presence of the OqxAB pump on susceptibility for selected compounds was investigated using broth dilution assays.**

***Results*: The OqxAB pump conferred antimicrobial resistance or reduced susceptibility towards a variety of substrates in *E. coli*. These included animal growth promoters, antimicrobials, disinfectants and detergents. pOLA52 could readily be transferred to enterobacterial pathogens. Transconjugants showed reduced susceptibility towards chloramphenicol, ciprofloxacin and olaquindox.**

***Conclusions*: The plasmid-encoded OqxAB pump has a wide substrate specificity and can be transferred between Enterobacteriaceae conferring reduced susceptibility to a multitude of substrates. These results could indicate some dependence on the outer membrane proteins present in the different species.**

Keywords: multidrug efflux pumps, antibiotic resistance, growth promoters, disinfectants, detergents

Introduction

In recent years, concern has been raised regarding the use of antimicrobials in animal production.[1] Previously, we have identified conjugative plasmids, such as pOLA52, which encode resistance to the quinoxaline compound olaquindox in *Escherichia coli* isolates of porcine origin from Denmark and Sweden.[2,3] The resistance mechanism was identified to be a multidrug efflux pump, OqxAB, which belongs to the resistance nodulation division (RND) family. OqxAB is one of the first plasmid-borne efflux pumps of the RND family and the first identified genetic resistance mechanism towards olaquindox. Furthermore, this pump confers resistance to chloramphenicol and ethidium bromide.[4] In this study, we investigated the substrate specificity for the OqxAB pump and the ability to function in other Enterobacteriaceae when pOLA52 is horizontally transferred.

Materials and methods

The following stock solutions were made: acriflavin, benzalkonium chloride, carbadox, cetrimide, ciprofloxacin, flumequine, norfloxacin, olaquindox, tetracycline and triclosan were dissolved in 0.5 M NaOH; chlorhexidine, H_2O_2, mitomycin C, nalidixic acid and SDS were dissolved in sterile H_2O; erythromycin, chloramphenicol and trimethoprim were dissolved in 96% ethanol and rifampicin was dissolved in 50% (w/v) methanol. The test range for all compounds included more than three dilutions on each side of the established MIC.

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The specificity of the OqxAB pump was tested in triplicate in 5 mL of Luria Bertani (LB) broth. For standardization of obtained MICs, 200 μL of overnight cultures of *E. coli* N43/pLOW2 and N43/pLOW2::*oqxAB*[4] was frozen in glycerol at 80°C. pLOW2::*oqxAB* (10–15 copies per cell) has previously shown similar MICs of olaquindox and chloramphenicol as wild-type pOLA52.[4] For each MIC measurement, one tube of each strain was used to inoculate 100 mL of LB broth and incubated overnight. Ten microlitres of 10 times diluted overnight cultures (2×10^6 cells) of the two strains was inoculated into 5 mL of LB broth containing 2-fold dilutions of the selected antimicrobials. Bacterial growth was measured as optical densities at 600 nm (OD_{600}) following incubation at 37°C overnight with 300 rpm shaking. The MIC was defined as the lowest concentration having an inhibitory effect of the OD_{600} of at least 90% when compared with the mean value of three controls.

In order to investigate the functionality of OqxAB pump in different pathogens, an Entranceposon cassette (Finnzymes, Finland) containing a kanamycin resistance [*npt* (KAN^R)] marker was inserted into the *bla* gene of pOLA52 according to the manufacturers' protocol using *E. coli* Genehogs® (Invitrogen). This mutagenized plasmid, pOLA52-*bla*::*npt*, was isolated and transferred by electroporation into *E. coli* CSH26.[5] On the basis of the growth requirement of CSH26 for proline, transconjugants could be isolated on minimal media without amino acid supplements when *E. coli* CSH26/pOLA52-*bla*::*npt* was used as donor strain in the subsequent mating experiment. No reduction was assumed in the conjugative abilities because of integration of the *npt* cassette. *Salmonella* Typhimurium DT27,[6] *Klebsiella pneumoniae* DSA712 (Dorthe Sandvang, Novozymes, Denmark), *Kluyvera* sp. MB101[7] and *Enterobacter aerogenes* DSM30053 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were used as recipients in conjugation experiments. All these strains used could grow on minimal media. Approximately 2×10^9 recipient and 2×10^8 donor cells were placed on a 0.2 μm filter on an LB agar plate and incubated overnight at 37°C. Cells were removed from the filter and plated onto minimal media containing 0.2% glucose and kanamycin. Transconjugants were isolated after 2 days on LB agar containing kanamycin and frozen for later use. MICs of chloramphenicol, ciprofloxacin or olaquindox were determined for both the recipients and their corresponding transconjugants using a broth dilution assay in microtitre plates.

Results and discussion

The MIC values measured here do not reflect the impact of the OqxAB pump on wild-type Enterobacteriaceae. To avoid interference from the major chromosomally encoded RND family pump in *E. coli*, the AcrAB pump, MICs were all measured in strain N43 containing a deleted *acrA* gene. MICs in wild-type *E. coli*, expressing both the OqxAB and AcrAB pumps, are expected to be higher than the ones measured here. MICs from an Entranceposon-mutagenized pOLA52 derivative (*npt* inserted in the *oqxB* gene) in *E. coli* N43 verified that the observed enhanced resistance was due to OqxAB activity and not due to plasmid-mediated overexpression of other chromosomally encoded RND pumps (data not shown).

MIC results are presented in Table 1. Reduced susceptibility towards selected chemicals was observed. Only a 4-fold difference was considered significant based on the experimental setup. As mentioned earlier, OqxAB conferred reduced susceptibility to the quinoxaline compound olaquindox and to chloramphenicol.[4]

Table 1. MICs of different chemicals for *Escherichia coli* N43 harbouring a plasmid with or without the *oqxAB* genes

Compound	MIC (mg/L)		
	N43/pLOW2	N43/pLOW2::*oqxAB*	increase[a]
Acriflavin	32	≥64	≥2×
Benzalkonium chloride	2	16	8×
Carbadox	8	64	8×
Cetrimide	2	8	4×
Chloramphenicol	2	256	128×
Chlorhexidine	0.5	1	2×
Ciprofloxacin	0.0078	0.125	32×
Erythromycin	32	32	—
Flumequine	0.25	8	32×
H_2O_2	2	2	—
Mitomycin C	4	8	2×
Nalidixic acid	8	64	8×
Norfloxacin	0.0313	1	64×
Olaquindox	8	256	64×
Rifampicin	16	16	—
SDS	512	65 536	128×
Tetracycline	0.25	0.5	2×
Triclosan	0.0078	0.0625	8×
Trimethoprim	0.25	8	64×

[a]Fold increase in resistance when harbouring a plasmid bearing the OqxAB pump.

Not surprisingly, we showed reduced susceptibility for carbadox, another quinoxaline. Reduced susceptibility was observed for quinolones (nalidixic acid) and fluoroquinolones (flumequine, norfloxacin and ciprofloxacin). Quinolone resistance has previously been related with several RND efflux pumps.[8] The OqxAB pump mediated reduced susceptibility towards trimethoprim. No effect was seen for tetracycline.

Apart from the growth-promoting and therapeutic antimicrobials, the OqxAB pump was also tested for its ability to reduce the susceptibility towards disinfectants or quaternary ammonium compounds (QACs) such as benzalkonium chloride and cetrimide. Reduced susceptibility was detected for benzalkonium chloride, whereas for cetrimide, no or a limited effect was detected (8- and 4-fold, respectively). An 8-fold reduced susceptibility was also observed towards the antibacterial compound triclosan, found in toothpaste, cosmetics and toys.[9] When testing the detergent SDS (an anionic surfactant), we observed a marked increase in tolerance (128-fold) in the OqxAB-positive strain. SDS is used in many household chemicals including shampoo and toothpaste. Although most of the OqxAB substrates have already been identified as substrates for other RND pumps,[8] noteworthy exceptions are quinoxalines and QACs.

Plasmid-borne multidrug efflux pumps encoding both resistance to antimicrobials and disinfectants could cause serious problems as not only usage of antimicrobials but also of compounds used in daily living will select plasmids encoding resistance to important antimicrobials for human treatment.

The efficacy of the RND-type multi-efflux pumps in Enterobacteriaceae is dependent on the presence of an outer membrane

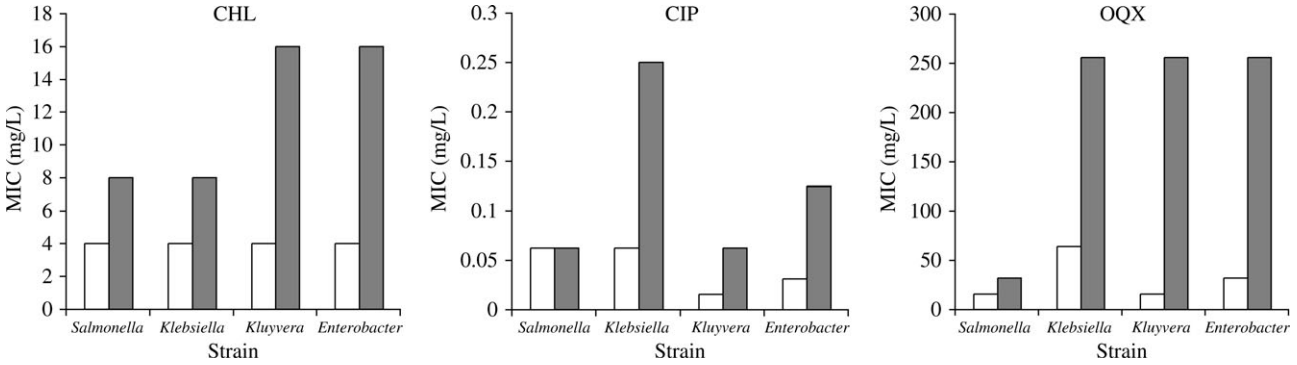

Figure 1. MICs of chloramphenicol (CHL), ciprofloxacin (CIP) and olaquindox (OQX) for *Salmonella* Typhimurium DT27, *Klebsiella pneumoniae* DSA712, *Kluyvera* sp. MB101 and *Enterobacter aerogenes* DSM30053 with or without pOLA52-*bla*::*npt*. White bars represent wild-type strains and grey bars represent the respective transconjugant strains harbouring pOLA52-*bla*::*npt*.

protein (OMP). In *E. coli*, the TolC protein participates.[10] Other Enterobacteriaceae also encode OMPs with high similarity to TolC. In *Salmonella*, the homology is 89% on the amino acid level.[11] To test the efficiency of the OqxAB pump, the pOLA52-*bla*::*npt* was conjugated to selected Enterobacteriaceae with conjugation frequencies ranging from 2.4×10^{-2} to 1.2×10^{-6} transconjugants/donor. Figure 1 shows the results obtained for these transconjugants. A clear difference in MIC was detected when comparing transconjugants with recipient strains, except for *Salmonella*. This could indicate a lower compatibility of the TolC homologue in *Salmonella*.

We have recently described OqxAB as one of the few efflux pumps encoded on conjugative plasmids.[2,4] Tauch *et al.*[12] have also published the sequence of a conjugative plasmid (pB4) bearing a different RND pump, MexCD, isolated from activated sludge. These findings of plasmid-encoded multidrug efflux pumps could indicate an emerging resistance problem. When we consider the variety of RND pump substrates,[8] it is obvious that several niches exist in which there is a selective pressure to maintain these plasmids.

Furthermore, we recently demonstrated that the pOLA52 plasmid facilitates biofilm formation on both biotic and abiotic surfaces in Enterobacteriaceae including *Salmonella* by the expression of type 3 fimbria (M. Burmølle, M. I. Bahl, L. B. Jensen, S. J. Sørensen and L. H. Hansen, unpublished results). Combined with the results obtained here, these identified efflux pumps could constitute a potential health hazard if transferred to pathogens. Therefore, in niches containing high bacterial numbers, such as the intestinal tract, the usage of antimicrobials for purposes other than treatment should be avoided.

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Transparency declarations

None to declare.

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