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MULTIDRUG EFFLUX SYSTEMS IN *ESCHERICHIA COLI* AND *ENTEROBACTER CLOACAE* OBTAINED FROM WHOLESOME BROILER CARCASSES

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ABSTRACT

Members of the Enterobacteriaceae family are present in the intestines of man and animals as commensals or are important disease causing agents. Bacteria bearing multidrug efflux systems (MDR) are able to survive adverse ecological niches. Multiresistant *Escherichia coli* and *Enterobacter cloacae* isolates from wholesome broiler carcasses were investigated for the presence of MDR. Lowering of Minimal Inhibitory Concentration for antimicrobials in the presence of a proton-motive force (PMF) uncoupler was tested as a potential display of the MDR phenotype. PCR amplification of the genes encoding AcrA and AcrB, components of a MDR system was performed. Diversity of each species was ascertained by Pulsed-Field Gel Electrophoresis (PFGE) of DNA digested with endonuclease *Xba*I. For all the isolates, except *E. coli* 1 and *E. cloacae* 9, lowering of MIC or of the growth rate in the presence of antimicrobials was observed, indicating a PMF dependent resistance mechanism. Expected products of DNA amplification with *acrAB* derived primers was obtained with all *E. coli* strains and with two of the five *E. cloacae* strains. Dendrogram generated shows diverse pulsetypes, confirming the genetic diversity among the strains. An important issue and related public health is the fact that different models and mechanisms of antimicrobial resistance are present in a small number of non-pathogenic strains and isolated from the same origin. These may be sources of resistance genes to others microorganisms, among them, pathogenic strains.

Key words: multiresistance; antimicrobials; Enterobacteriaceae; proton-motive force; diversity

INTRODUCTION

Antimicrobial agents have been broadly used in modern avian industries even though this practice is now challenged or banned in many countries (2,6). Their use has spanned objectives such as prevention of diseases, growth promoting activities, and therapeutics (2). This practice has possibly caused favorable conditions for the selection, distribution, and persistence of antimicrobial-resistant bacteria (1). Commensal bacteria exposed to antimicrobial agents may have had their populations selected for a resistance trait and thus become a reservoir for potentially mobile resistance genes, capable of being transferred to pathogenic strains (3). Multidrug efflux systems (MDR) have commanded attention because a single resistance mechanism may diminish susceptibility to several therapeutic drugs (13,25), allowing the survival of bacteria in their niches (23). There are currently five known families of proteins with the MDR phenotype. One of them, the Resistance Nodulation Cell Division (RND) family, is expressed in Gram-negative bacteria, and is linked to clinically significant drug resistance (23), driven by

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proton-motive force (11). The AcrAB efflux system belongs to the RND family and is one of the primary efflux systems associated with *E. coli*; its overexpression has been reported in clinical isolates (23). Primers derived from *E. coli* acrAB sequences yielded positive results for PCR amplification of DNA from different strains of *E. cloacae* (19). *E. coli* and *E. cloacae* are important commensals or pathogens that inhabit the gastrointestinal tract of humans and animals (10,12), and are thought to be important sources of antimicrobial resistance genes for both animal and human pathogens (27,30). A single population of bacteria may present different degrees of genetic diversity and also different antimicrobial resistance mechanism models.

In this study, we report the putative presence of MDR-dependent antimicrobial resistance in non-pathogenic *E. coli* and *E. cloacae* in genetically diverse strains isolated from broiler carcasses. The results bear ecological relevance and were obtained at a time when growth-promoting antimicrobials, such as spectinomycin and apramycin, were still in use, a practice which is still allowed in Brazil.

MATERIAL AND METHODS

Bacteria

Five *E. coli* and five *E. cloacae* strains were chosen from a collection of likely commensal isolates obtained from carcasses of clinically healthy broiler. Sampling was done on the processing line immediately after evisceration at an industrial slaughter facility. The isolates displayed distinct multi-resistance phenotypes and included in their resistance profiles the antimicrobials spectinomycin, apramycin, nalidixic acid, cefadroxil, cephalexin, cefaclor, furazolidone, nitrofurantoin, spiramycin, tetracycline, chloramphenicol, and sulfamethoxazole/trimethoprim. All strains were stocked at -80ºC in Brain and Heart Infusion, BHI (Oxoid, Basingstone, Hampshire, England), with 20% glycerol.

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis was performed according to Chang and Chui (7) with slight modifications.

For each strain, the cell-agarose plugs were prepared with 10^8 CFU/mL grown on BHI medium (Oxoid). For DNA hydrolysis, the plugs were treated with 50 units of *Xba*I restriction enzyme (Promega, Madison, Wisconsin, USA). The plugs were one millimeter thick. Phage Lambda concatamers (Sigma, St. Louis, Missouri, USA) were used as molecular size standards. DNA fragments were separated by PFGE using the CHEF-Dr III system (Biorad laboratories) for 17 h at 12ºC with an initial pulse of 2 s and a final pulse of 20 s; angle 120º; at 6 V/s. The agarose gel was stained with ethidium bromide and photographed under ultraviolet illumination.

The PFGE pulsetypes were compared by analyzing TIFF files with Gel Pro Analyser® 3.1 (Media Cybernetics Inc). Cluster analysis of the Dice Similarity Indexes, based on the unweighted pair group method using arithmetic averages (UPGMA), was carried out with the GENES program (9) to generate a dendrogram describing the relationship among the pulsetypes.

Minimal inhibitory concentration - MIC

The *E. coli* isolates were reactivated in BHI broth and incubated at 37ºC for 16-18 h. The cultures were diluted in BHI broth to 10^5 cfu/mL. Aliquots of 230 µl of the diluted culture were added to microtiter plate wells (Nunc-Immuno™) with various amounts of different antimicrobials (Sigma) for a total volume of 300 µl per well. The assays were carried out twice in triplicate, as were the required controls. Growth was periodically assessed spectrophotometrically in an ELISA plate reader (Titertek multiskan®, Plus-MkII) at λ540 nm. Curves were plotted with the media of the triplicates. The MIC was arbitrarily established as the minimum concentration of each antimicrobial that completely inhibited growth of the cells until two hours after the begining of the stationary phase of the control (*E. coli* growing in BHI) (18,19).

Active efflux phenotype

The presence of active efflux for each antimicrobial was determined by comparison of the MIC in the presence and absence of carbonyl cyanide m-chlorophenylhydrazone -CCCP (Sigma) (18,20). The highest CCCP concentration that would not affect the growth rate of each isolate was determined and used in the experiments.

PCR amplifications of the *acrA* and *acrB* genes

Specific primers for amplification of the genes *acrA* and *acrB* were constructed using *E. coli* K12 genetic sequences deposited in Gene Bank (accession nº M94248). The primer sets were 5'GGTCGTTCTGATGCTCTCA3' (forward) e 5'GGCTTGCT GGTTATTATCAG3' (reverse) for *acrA*, and 5'CGTCTAACAG TGACTCCACGG3' (forward) and 5'TTCAATCAGACC TTTACCTTC3' (reverse) for *acrB*, and were synthesized by Life technologies, Gibco BRL (São Paulo, SP, Brazil).

DNA amplification was carried out in a 25 µl volume containing high temperature lysed colony cells, 25 mM $MgCl_2$ (Promega), 2.5 mM each of the four dNTP(s), and 1 unit Taq DNA polymerase (Promega) in the proper buffer (Promega). The thermocycler PTC-100 (MJ Research) was programed for 40 cycles consisting of 94ºC/1 min; 52ºC/ 1 min; 72ºC/2 min. The last cycle was followed by 7 min at 72ºC. To amplify a large fragment of approximately 2730 bp, the extension time at 72ºC was 3.5 min, and 1.7 units of Taq DNA polymerase were used. Negative controls were performed in all cases, as well as a positive control with *E. coli* K12. The products were analysed by agarose gel electrophoresis on 1% and 1.2% agarose depending on the expected amplicon length.

RESULTS

Genetic diversity among *Escherichia coli* and *Enterobacter cloacae*

The genetic diversity of *E. coli* and *E. cloacae* isolated from the same environment, poultry carcasses, was assessed by PFGE (Fig. 1) The restriction profiles generated from *Xba*I hydrolysis of the total DNA of four *E. coli* and five *E. cloacae* strains were all distinct. The considered restriction fragments varied from about 50 kb to approximately 400 kb. The resulting dendrogram separated *E. coli* strains from *E. cloacae* except for *E. coli* 2, which was positioned among an *E. cloacae* cluster. All the isolates were identified by morphotinctorial and biochemical tests. Both somatic and flagellar antigens were nontyping for *E. coli* 2. For technical reasons, *E. coli* 6 was not included in the dendrogram.

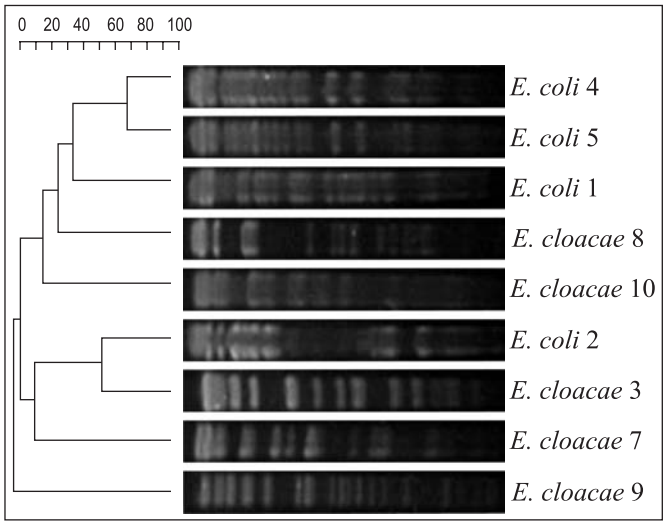

Figure 1. Dendrogam generated by Genes program, showing distances calculated by the Dice similarity of PFGE *Xba*I patterns among 9 pulsetypes. The degree of similarity (%) is shown on the scale.

Antimicrobial resistance profiles of *E. coli* and *E. cloacae*

Table 1 shows the minimal inhibitory concentrations (MICs) of the *E. coli* and *E. cloacae* strains. Twelve antimicrobial drugs, encompassing several chemical classes, were chosen from among those used as growth promotion agents and as therapeutic drugs for broiler. The resulting MICs were determined only for the antimicrobials for which each strain was found resistant. The resistance profiles were strikingly different and correlated with the genetic results.

Effect of CCCP on resistance to antimicrobials

Table 2 shows the resistance profiles of *E. coli* and *E. cloacae* when cells were treated with the maximum concentration of CCCP, an energy uncoupler that does not affect the growth of each particular strain. For all isolates, this concentration was 20 µM. Only five strains, *E. coli* 2, *E. coli* 4, *E. coli* 6 and *E. cloacae* 10, displayed lower MICs for some antimicrobials when CCCP was present, as compared to Table 1.

E. coli 2 was more sensitive to furazolidone, nitrofurantoin, spiramycin, tetracycline, chloramphenicol, and sulfamethoxazole/ trimethoprim *E. coli* 4 and *E. coli* 6 had reduced MICs for nitrofurantoin and sulfamethoxazole/

Table 1. Minimal Inhibitory Concentrations, in BHI broth, of selected antimicrobials on *Escherichia coli* and *Enterobacter cloacae*.

Isolates	Antimicrobials (µg/ml)											
	S	APR	NA	CFR	CEC	CL	FR	F	SP	TE	C	SXT
E. coli 1						30		50				
E. coli 2				20		20	50	70	500	200	30	2000
E. coli 4	250		800			20		80			30	1500
E. coli 5			450	20							20	
E. coli 6	60	1400				20		40		2.5		1800
E. cloacae 3						30			900			
E. cloacae 7				30		30			700			
E. cloacae 8	30		10	10		20			400	2.5		
E. cloacae 9						30			1000			
E. cloacae 10	20			10	5							

S: spectinomycin; APR: apramycin; NA: nalidixic acid; CFR: cefadroxil; CEC: cefaclor; CL: cefalexin; FR: furazolidone; F: nitrofurantoin; SP: spiramycin; TE: tetracycline; C: chloramphenicol; SXT: sulfametoxazol/trimetoprim and blank space: sensitive.

trimethoprim; *E. cloacae* 10 had a diminished MIC for cefaclor. These results indicate energy-dependent resistance mechanisms for these drugs among those isolates. However, even though MICs for other antimicrobials or for other strains were not lowered in the presence of the uncoupler, the method used made it possible to observe that the growth of some strains was particularly impaired by some antimicrobials in the presence of CCCP (data not showed). Asterisks mark those MICs (Table 2).

Presence of the *acrA* and *acrB* genes in *Escherichia coli* and *Enterobacter cloacae*

Primers for amplification of *acrA* amplified fragments of expected length, about 1.1 kb, in *E. coli* K12, the positive control. Primers for *acrB* amplified a fragment of an approximately 2.7 kb, as expected. Results of *acrA* and *acrB* amplification are displayed in Fig. 2A and B, respectively. As expected, all *E. coli* were positive for these genes. Among the *E. cloacae* strains,

Table 2. Effect of 20mM CCCP on the Minimal Inhibitory Concentration, in BHI broth, of selected antimicrobials on *Escherichia coli* and *Enterobacter cloacae.*

Isolates	Antimicrobials (μg/ml)											
	S	APR	NA	CFR	CEC	CL	FR	F	SP	TE	C	SXT
E. coli 1						30		50				
E. coli 2				20		*20	**20**	**≤40**	**450**	**150**	**20**	**≤1900**
E. coli 4	250		*800			20		**≤50**			30	**1100**
E. coli 5			*450	20							*20	
E. coli 6	*60	1400				*20		**30**		2,5		**1600**
E. cloacae 3						*30			900			
E. cloacae 7				*30		*30			700			
E. cloacae 8	30		*10	10		*20			400	*2,5		
E. cloacae 9						30			1000			
E. cloacae 10	*20			*10	**≤2,5**							

CCCP: carbonyl cyanide m-chlorophenylhydrazone; S: spectinomycin; APR: apramycin; NA: nalidixic acid; CFR: cefadroxil; CEC: cefaclor; CL: cefalexin; FR: furazolidone; F: nitrofurantoin; SP: spiramycin; TE: tetracycline; C: chloramphenicol; SXT: sulfametoxazol/ trimetoprim; ***:** isolates that presented lowering of growth rates without changing MIC; blank space: sensitive and; boldface: MIC reduced in the presence of CCCP.

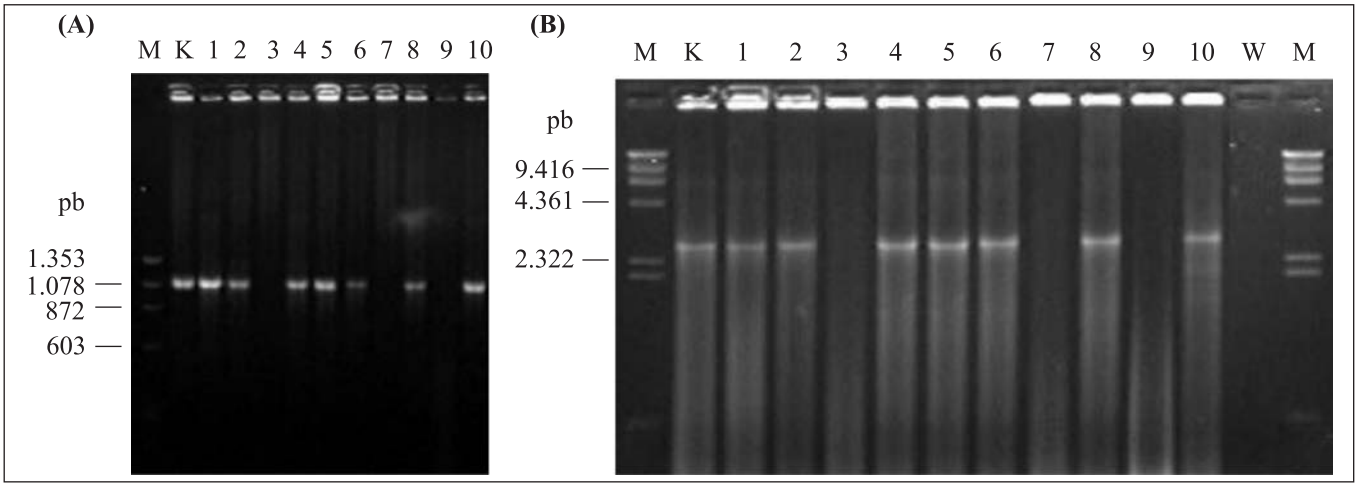

Figure 2. PCR products of *ACrA* (A) and *AcrB* (B) amplification of *Escherichia coli* (lines 1, 2, 4, 5 and 6) and *Enterobacter cloacae* (lines 3, 7 and 9) DNA. M: size marker, phage ϕX174/*HaeIII* DNA (A) and phage λ/*HindIII* (B); K: positive control, *E coli* K12 DNA PCR product; W: reaction mixture, no template (B).

only strains 8 and 10 presented positive results, consistent for both genes.

DISCUSSION

The dendrogram generated from PFGE profiles (Fig. 1) demonstrates that the four isolates of *E. coli* are distinct from each other, as are the five *E. cloacae* isolates. Although the *Xba*I restriction endonuclease discriminated most of the isolates, i.e., *E. coli* and *E. cloacae*, this was not true for *E. coli* 2. This strain was placed closer to the *E. cloacae* strains than to the other *E. coli*. These two species are phylogenetically very closely related among the Enterobacteriaceae family and they obviously have the same habitat, so one test alone may not be sufficient to discriminate between some of the strains (8,31). Polyphasic taxonomy takes into account phenotypic, ecological and genetic data and integrates all information to yield a more precise and trust worthy identification and classification of the strains (8).

The diversity among individuals in the isolated groups was also observed by their resistance profiles and the different MICs before (Table 1) and after adding CCCP (Table 2) for each antimicrobial tested. The different resistance levels observed among the isolates suggests diverse combinations of strategies. This would pose a problem because bacteria of the same source could be acquiring different resistance genes for the same antimicrobial, thus diversifying the pool of resistance determinants. *E. coli* 2, a multiresistant strain, had a completely distinct resistance profile when compared to all *E. coli* and *E. cloacae* strains tested.

All the antimicrobial agents were represented at least once in the resistance profiles of the strains. Apramycin, cefaclor and furazolidone appeared only once in the resistance models (Table 1). Cefalexin resistance was the most prevalent, being present in eight isolates, followed by resistance to cefadroxil and espiramycin, both represented five times (Table 1). Cefalexin and cefadroxil are both beta-lactams and first generation cephalosporins (16). Cefaclor is a second generation cephalosporin (16); it is understandable why more strains are resistant to those drugs, which have been used for a longer time. The most frequent mechanism of resistance to beta-lactam is possibly the presence of beta-lactamases, enzymes that hydrolyze the amide bond at the beta-lactam ring (16). If this is the case, there is also a diversity of beta-lactamases because the resistance profiles did not coincide in all isolates when this class of antimicrobials was considered. The lowering of the MIC or of growth rates in the presence of the antimicrobial drugs and the uncoupler CCCP for all isolates, except for *E. coli* 1 and *E. cloacae* 9 (Table 2), suggest a PMF-dependent resistance mechanism. *E. coli* 2 is a good candidate for MDR studies (Table 2) and diversity (Fig. 1).

As expected, oligonucleotide primers designed to amplify genes of the *acrAB* operon yielded positive results with all the *E. coli* isolates (Fig. 2A and B). The AcrAB multidrug efflux system is the main efflux pump in *E. coli* and it is responsible for the acquisition of multiple antimicrobial resistance, including resistance to tetracycline, chloramphenicol, fluoroquinolones, fusidic acid, lipophilic beta-lactams antibiotics, nalidixic acid, novobiocin and rifamicin (21,22). *E. coli* 2, 4, 5 and 6 displayed PMF-dependent resistance mechanisms as far as nalidixic acid, tetracycline, cefalexicin and chloramphenicol were concerned (Table 2). PMF is the source of energy for the transport proteins AcrA and AcrB (11). It is possible that in the tested isolates, the AcrAB system was responsible for the PMF-dependent resistance observed.

Among the five *E. cloacae* isolates, two (isolate numbers 8 and 10) tested positive by PCR for both genes *acrA* and *acrB* (Fig. 2A and 2). The efflux system AcrAB has been identified in *E. cloacae* from human sources (14) and from poultry sources (19). The genes encoding *acrAB-tolC* efflux pumps were identified in the nosocomial pathogen *Enterobacter aerogenes* (26). An increased in AcrA protein was observed in clinical isolates of imipenem-resistant *E. cloacae* (4). *E. cloacae* 8 and 10 displayed PMF-dependent resistance to nalidixic acid, tetracycline, cefadroxil, cefalexin, and cefaclor (Table 2). These antimicrobials are substrates of the AcrAB multidrug efflux system. Besides the known substrates of the AcrAB system, other antimicrobials were found to possess some PMF-dependent resistance mechanisms (Table 2). Phenotypic and genotypic tests revealed the possible presence of the AcrAB system (Table 2, Fig. 2A and 2B). This system could possibly transport other antimicrobials of different classes, such as spiramycin, furazolidone, nitrofurantoin, sulphametoxazol/trimetoprim (Table 2), or there may be other systems functioning simultaneously. DNA sequencing and mutant analysis may bring some light to this issue. Amplifications of AcrA or AcrB was observed in *E. coli* 1 (Fig. 2A and B), but the PMF-dependent resistance phenotype was not detectable (Table 2). Synergism among resistance mechanisms has been reported (24) and the AcrAB system is constitutively expressed (17). So, it is possible that this MDR could be functional but at very low levels that are undetectable by the methods used here.

While many studies have evaluated resistance in bacterial pathogens, relatively few have looked at commensal bacteria. These bacteria are present in the agricultural environment in numbers far greater than pathogens; they are major players in the harboring and disseminating of antibiotic resistance genes (1,28). Such a diversity of resistance models and possible resistance mechanisms detected in so few isolates from the same source not related to pathogenic serotypes may indicate an important public health issue concerning commensal bacteria in food animals. *E.coli* and *E. cloacae* are inhabitants of the gastrointestinal tract of humans and animals (10,12). While *E. coli* is widely studied as a food pathogen, *E. cloacae* has emerged as an important nasocomial pathogen with numerous

outbreaks of infections being reported (15,27). Bacteria of animal origin may reach human beings through several routes and genetic determinants of resistance may transfer to the human microbiota and diffuse to different geographic areas (5,29).

The five *E. coli* isolates and six *E. cloacae* isolates, apparently a small sample, were all different strains, a fact that stresses the ample diversity among these bacteria as far as drug resistance mechanisms are concerned.

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RESUMO

Sistemas de efluxo multidroga em *Escherichia coli* e *Enterobacter cloacae* obtidas de carcaças de frangos sadios

Os membros da família Enterobacteriaceae estão presentes no intestino do homem e dos animais como comensais ou agentes causadores de doença importantes. Bactérias multirresistentes podem possuir sistemas de efluxo multidrogas (MDR) sendo capazes de sobreviver em nichos ecológicos adversos. *Escherichia coli* e *Enterobacter cloacae*, multirresistentes, isoladas de frangos sadios foram investigadas quanto à presença de MDR. A diminuição da concentração inibitória mínima de antimicrobianos, na presença de um desacoplador da força próton motora (PMF), foi usada para detectar o fenótipo MDR. Foi realizada PCR dos genes codificadores de AcrA e AcrB, componentes de um sistema MDR. A diversidade de cada isolado foi confirmada por eletroforese em gel de campo pulsado (PFGE) usando a endonuclease *Xba*I. Observou-se em todos os isolados, exceto *E. coli* 1 e *E. cloacae* 9, uma diminuição das MICs ou das curvas de crescimento na presença dos antimicrobianos, indicando um mecanismo de resistência dependente da PMF. Os produtos amplificados esperados derivados de *acrAB* foram obtidos em todos os isolados de *E. coli* e em dois, dos cinco, de *E. cloacae*. O dendrograma gerado mostra diferentes perfis de bandas (pulsetypes), confirmando a diversidade genética entre os isolados. Uma questão importante e relacionada à saúde publica é o fato de que diferentes modelos e mecanismos de resistência aos antimicrobianos estão presentes em um número reduzidos de isolados não patogênicos e obtidos de uma mesma origem. Esses podem ser fontes de genes de resistência para outros microorganismos, entre eles, cepas patogênicas.

Palavras-chave: multirresistência; antimicrobianos; Enterobacteriaceae; força próton motora; diversidade

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