Effects of chlorophyll-derived efflux pump inhibitor pheophorbide *a* and pyropheophorbide *a* on erythromycin resistance of *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella* Typhimurium and *Escherichia coli*

Mareike Kraatz[1], Dr. vet. med., Terence R. Whitehead[1], Ph. D., Michael A. Cotta[1], Ph. D., and Mark A. Rasmussen[2], Ph. D.

[1] National Center for Agricultural Utilization Research, ARS, USDA; Peoria, IL, USA; [2]Leopold Center for Sustainable Agriculture, Iowa State University; Ames, IA, USA

INTRODUCTION - OBJECTIVES

Recently, it was hypothesized[1] that pheophorbide *a* (Php) and possibly other dietary chlorophyll *a* catabolites (Fig. 1) with drug efflux pump inhibitor (EPI) activity may reverse antimicrobial resistance of gastrointestinal (GI) bacteria in livestock.

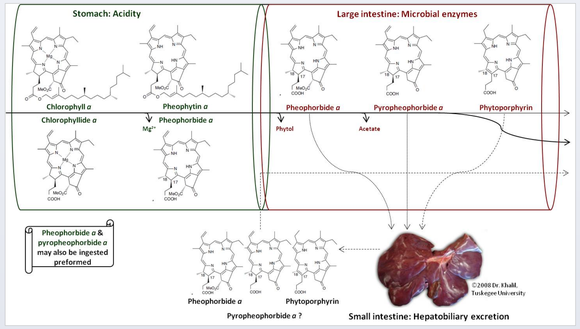


Fig. 1: Enterohepatic metabolism of dietary chlorophyll *a* and its catabolic derivatives in humans and non-ruminant livestock[2-5]

In the present study, we investigated the *in vitro* effects of Php and pyropheophorbide *a* (Pyr) on erythromycin resistance and growth kinetics of selected pathogenic and opportunistic indicator bacteria with macrolide or multidrug resistance (MDR) efflux pumps (Fig. 2).

INTRODUCTION - BACKGROUND

Erythromycin in human medicine and agriculture

Erythromycin is currently FDA-approved for human and veterinary use. It belongs to the macrolide class of antibiotics which is critically important in human medicine[6,7]. Its use has diminished over time due to increased bacterial resistance, but it is still an important alternative against human respiratory and food-borne infections[8]. Erythromycin can be applied in medicated feed for swine, cattle and poultry[9]. It is also routinely used by the corn ethanol industry, and inadvertent exposure of livestock to residues in distillers by-products is a growing concern[9]. The related macrolide tylosin, which is estimated to be the quantitatively second most common in-feed antimicrobial in the U.S. swine production[7], can spur bacterial resistance to erythromycin[10].

Efflux-mediated erythromycin and multidrug resistance

Erythromycin is a narrow-spectrum antibiotic, as it is effectively extruded by MDR pumps in many Gram-negative bacteria[11]. In Gram-positive bacteria, efflux is the main mechanism of erythromycin resistance besides rRNA target modification[12]. Efflux pumps are a major contributor to bacterial MDR[13], and efflux-mediated MDR is an increasing clinical problem due to its rising prevalence, especially in Gram-positive human pathogens[14].

What the literature says

EPIs from natural sources to overcome MDR

A wide array of plant compounds with EPI activity has been identified in recent years, however, i. a. due to intrinsic toxicity, so far no EPI/antimicrobial drug combination is used clinically[14,17]. The nutritional or co-therapeutic application of EPIs is a promising means of reversing MDR and mitigating its transmission through enhanced antimicrobial activity and colonization prevention[11,13,17].

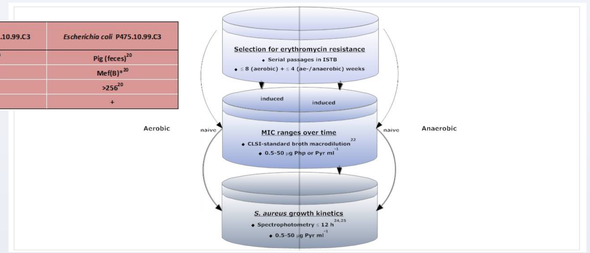
Animal-to-human transmission of MDR

Transmission of antimicrobial resistance and especially MDR from livestock bacteria to human pathogens presents a public health risk and accounts for antimicrobial resistance being one of the major global public health challenges of today and the future[6]. It can occur directly, such as in the case of livestock-associated meticillin-resistant *Staphylococcus aureus* (LA-MRSA)[15], or via horizontal transfer of genetic resistance determinants in (e.g. soil) microbiota in contaminated habitats[16].

MATERIALS & METHODS

Characteristic	*Enterococcus faecalis* ATCC 29212	*Staphylococcus aureus* ATCC 29213	*Salmonella enterica* serovar Typhimurium ATCC 14028	*Escherichia coli* P286.10.99.C3	*Escherichia coli* P475.10.99.C3
Isolation	Human (urine)[18]	Human (wound)[14]	Chicken (heart, liver)[19]	Pig (feces)[20]	Pig (feces)[20]
Multidrug or macrolide* efflux pump	EmeA[21]	NorA/B/C, MdeA, LmrS, Mef(A)+[21]	AcrAB-TolC[21]	Mef(B)+[20]	Mef(B)+[20]
Erythromycin MIC (µg/ml)	2-256[21,23]	0.25-0.5[2123]	128->256[23]	>256[20]	>256[20]
Erythromycin resistance	-/+	-	+	+	+

Fig. 2: Characteristics of bacterial reference strains and overview of methods used in this study



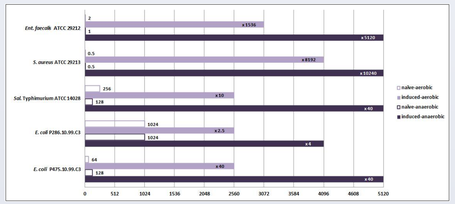


Fig. 3: Overall maximum tolerated concentration of erythromycin (µg ml^{-1}) and induction factor

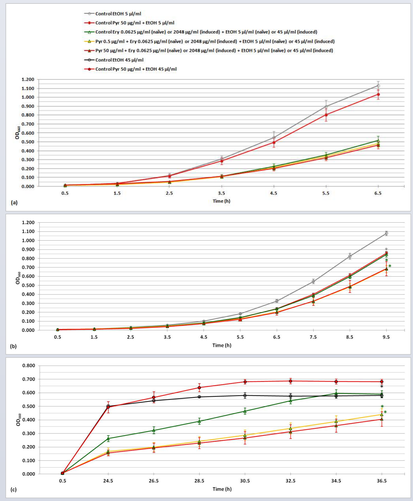


Fig. 5: Growth curves of naïve (a, b) and induced (c) *S. aureus* ATCC 29213 under aerobic (a) and anaerobic (b, c) conditions. Values are averages and standard deviations from three repetitions.

RESULTS

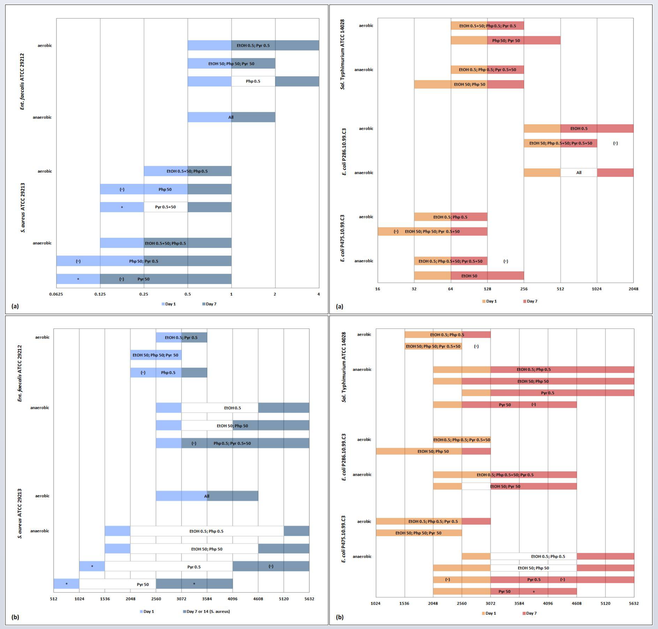


Fig. 4: Initial (day 1) and final (day 7 or 14) MIC ranges of erythromycin (µg ml^{-1}) for naïve (a) and induced (b) Gram-positive and -negative reference strains. Values are from three repetitions.

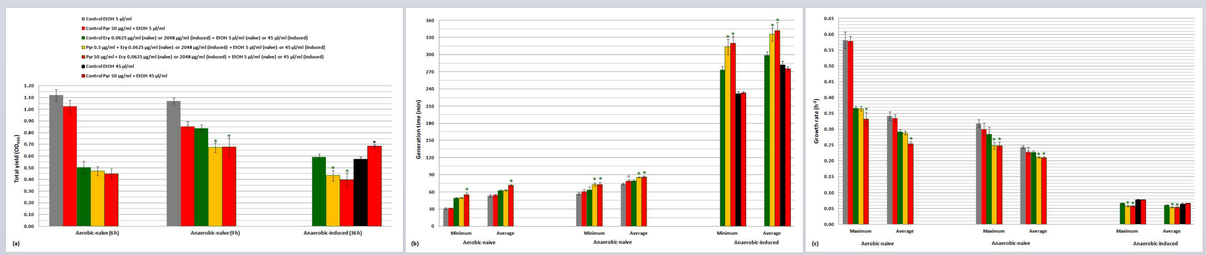


Fig. 6: Growth parameters of *S. aureus* ATCC 29213. Values are averages and standard deviations from three repetitions.

CONCLUSIONS

Pyr, but not Php, potentiated the antibiotic effect of erythromycin against erythromycin-naïve (susceptible) *S. aureus* ATCC 29213 and partially reversed erythromycin resistance of erythromycin-induced (highly resistant) *S. aureus* ATCC 29213.

Its effect was concentration-dependent (50 µg ml^{-1} > 0.5 µg ml^{-1}) and presumably due to inhibition of MDR or macrolide efflux pump(s) other than NorA [e.g. NorB/C, MdeA, LmrS, Mef(A)]. It was greatest in the highly resistant strain and under anaerobiosis.

Pyr exerted an intrinsic inhibitory effect against erythromycin-susceptible *S. aureus* ATCC 29213 under anaerobic conditions. This indicates that Pyr interacts with a metabolically regulated MDR pump with broader physiological functions.

Effects of Pyr and Php on strains of *Ent. faecalis*, *Sal.* Typhimurium and *E. coli* were less consistent (*E. coli* P475) or not significant.

This is the first study indicating that chlorophyll-derived Pyr can reduce antibiotic resistance and growth of bacteria in anaerobic habitats, such as the GI tracts and wastes of livestock.

ACKNOWLEDGEMENTS

We thank Virve I. Enne, Centre for Immunology and Infectious Disease at Barts and The London School of Medicine and Dentistry, London, UK, for providing *E. coli* strains.

REFERENCES

1. Barnes, C. A., Rasmussen, S. L., Petrich, J. W. & Rasmussen, M. A. (2012). J Agric Food Chem 60(12): 10456-10460.
2. Ferruzzi, M. G. *et al*. (2001). J Agric Food Chem 49(4): 2082-2089.
3. Jonker, J. W. *et al*. (2002). PNAS 99(24): 15649-15654.
4. Ashby, K. D. *et al*. (2003). J Agric Food Chem 51(11): 3502-3507.
5. Campbell, W. M. *et al*. (2010). N Z Vet J 58(3): 146-154.
6. Marshall, B. M. & Levy, S. B. (2011). Clin Microbiol Rev 24(4): 718-733.
7. Apley, M. D. *et al*. (2012). Foodborne Pathog Dis 9(3): 1-8.
8. Jaglic, Z. *et al*. (2012). Zoonoses Public Health 59(3): 202-211.
9. Olmstead, J. (2012). Institute for Agriculture and Trade Policy. Minneapolis, MN.
10. Jacob, M. E. *et al*. (2008). J Anim Sci 86(5): 1182-1190.
11. Piddock, L. J. V. (2006). Nat Rev Microbiol 4(8): 629-636.
12. Aakra, A. *et al*. (2005). Antimicrob Agents Chemother 49(6): 2246-2259.
13. Li, X. Z. & Nikaido, H. (2009). Drugs 69(12): 1555-1623.
14. Stavri, M. *et al*. (2007). J Antimicrob Chemother 59(6): 1247-1260.
15. O'Brien, A. M. *et al*. (2012). PLoS ONE 7(1): e30092.
16. Forsberg, K. J. *et al*. (2012). Science 337(6098): 1107-1111.
17. Abreu, A. C. *et al*. (2012). Nat Prod Rep 29(9): 1007-1021.
18. ATCC: The Global Bioresource Center (2012). http://www.atcc.org/ATCCAdvancedCatalogSearch.aspx .
19. Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (2012). http://www.dsmz.de/catalogues/details/culture/DSM-19587.html
20. Liu, J. H., Keelan, P., Bennett, P. M. & Enne, V. I. (2009). J Antimicrob Chemother 63(3): 423-426.
21. Poole, K. (2005). J Antimicrob Chemother 56(1): 20-51.
22. Andrews, J. M. (2001). J Antimicrob Chemother 48: 5-16.
23. TOKU-E (2012). http://antibiotics.toku-e.com/antimicrobial_599.html.
24. Lindqvist, R. (2006). Appl Environ Microbiol 72(7): 4682-4870.
25. Madigan, M. T. *et al*. (2008). Brock Biology of Microorganisms, 12th international edn. San Francisco, CA: Pearson Education.

Contact information: Mareike.Kraatz@ARS.USDA.gov