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Exploration of Combinatorial Therapy for Gram Negative Bacteria such as Escherichia coli

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Exploration of Combinatorial Therapy for Gram Negative Bacteria such as *Escherichia coli*

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Microbiology, Immunology, and Molecular Genetics

by

Manzhu Kang

2014

ABSTRACT OF THE THESIS

Exploration of Combinatorial Therapy for Gram Negative Bacteria such as *Escherichia coli*

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Master of Science in Microbiology, Immunology and Molecular Genetics

University of California, Los Angeles, 2014

Professor Jeffrey H Miller, Chair

Antibiotic resistance is certainly a worldwide concern, which presents tremendous challenge and threat to public health management of infectious diseases. The development of new drugs has become a declining strategy to combat resistance, due to the protracted and circuitous nature of it, from the search of new antimicrobial agents to their approvals in clinical use. Therefore, many have turned to the rational approach of finding combinatorial therapies with existing drugs. In this thesis, I will explore combinatorial therapy for treatment of Gram-negative bacteria, such as *Escherichia coli*. We utilized a mutant strain, which lacks the enzyme of deoxycytidine deaminase, and showed its hypersensitivity to a

variety of compounds, from specific nucleotides to several antibiotics including vancomycin. We identified the synergistic interactions between vancomycin with cytidine, and with trimethoprim. The latter led us to the discovery of the striking potency of vancomycin and trimethoprim even in the wild-type strain. This result presents valuable implication of expanding the spectrum of a Gram-positive antibiotic to the treatment of Gram-negative infections.

The thesis of Manzhu Kang is approved.

Catherine F Clarke

Robert P Gunsalus

Jeffrey H Miller, Chair

University of California, Los Angeles

2014

TABLE OF CONTENTS

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Chapter 2 is a version of Kang TM, Yuan J, Zhou A, Beppler C and Miller JH (2014) "Deoxycytidine Deaminase-deficient *Escherichia coli* Strains Display Acute Sensitivity to Cytidine, Adenosine, and Guanosine, and Increased Sensitivity to a Range of Antibiotics, Including Vancomycin." J. Bacteriol. 196(11):1095.

Chapter 3 includes a substantial portion of Zhou A, Kang TM, Yuan J, Beppler C, Nguyen C, Mao ZY, Nguyen MQ, Yeh P, and Miller JH (2014) "Synergistic Interaction of Vancomycin with Different Antibiotics in *Escherichia coli*: Trimethoprim and Nitrofurantoin Display Strong Synergies with Vancomycin in Wild-type *E.coli*" AAC. Manuscript submitted.

Individual Contributions to this Work

Chapter 2 – Jessica Yuan assisted in the establishment of sensitivity profile of *dcd* mutant. Casey Beppler and Alice Zhou assisted in performing the synergy experiment and kill assay.

Chapter 3 – Jessica Yuan assisted in the discovery of synergy between vancomycin and trimethoprim. Casey Beppler and Caroline Nyugen assisted in the pair-wise drug interaction study and the kill assay.

Chapter 1 - A Review of Antibiotic Resistance and Combinatorial Treatment Strategy

Infectious diseases are one of the leading causes of death worldwide (1), despite of the extraordinary accomplishments with the development and usage of antimicrobial agents. The infamous statement from then Surgeon General William H Stewart "It is time to close the book on infectious diseases" back in the 1960s (2), was simply wrong. One of the reasons for infectious diseases to remain as a battle to fight is its continual evolution of emerging and reemerging (3) where multidrug-resistant pathogens play a major role. In this chapter, I will review common mechanisms of antimicrobial resistance and strategies to combat.

The threat we face

CDC estimated more than two million illnesses and at least 23,000 deaths as a result of antibiotic-resistant infections every year in the United States in their "Threat Report" of 2013. (4) The scope of the problem is certainly not statewide but worldwide, giving the remarkable speed resistance can spread across continents. Gram-negative infections, among all, are particularly catastrophic due to their resistant to nearly all readily usable drugs.

Resistant Mechanisms

The development of resistance to antimicrobial agents has been in chronological parallel with the development and usage of drugs, as indicated in

Figure 1-1. (5, 6) In fact, the leading factor to resistance is the use and misuse of antibiotics. (7)

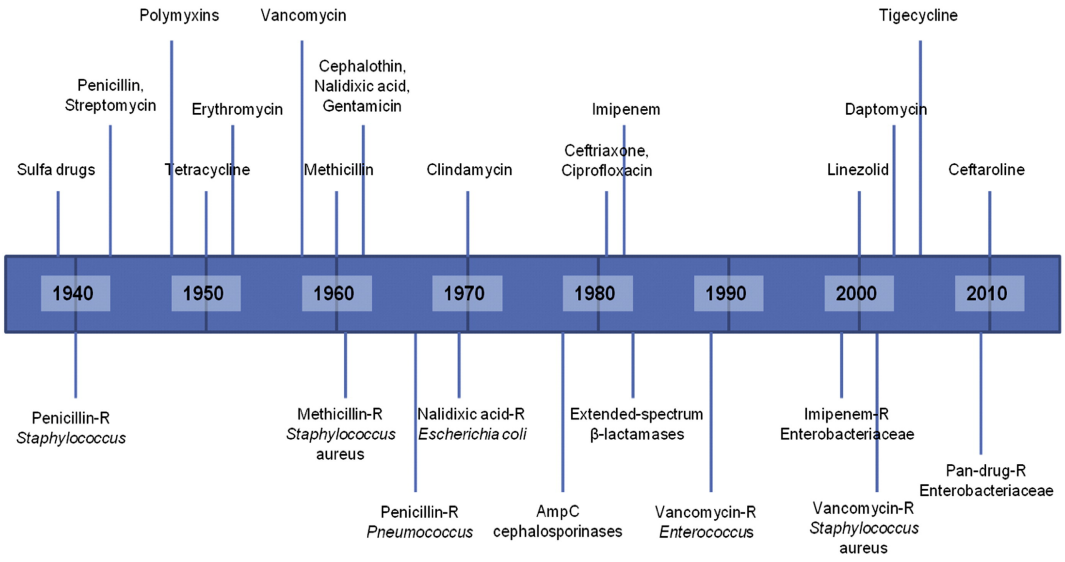

FIG 1-1 Timeline of antimicrobial drug discovery against the development of drug resistance. (From (5))

Resistance can be acquired through two different routes, in terms of its origin. First is the *de novo,* step-wise progression from low-level to high-level resistance through chromosomal mutations. (7, 8, 9) The second is acquisition of mobile resistant elements among taxonomically different bacteria by bacteriophages, plasmids, naked DNA or transposons (7,10). Common resistant mechanisms have been studied extensively and they generally fall into three different categories. First is targeting the antibiotic itself. A classic example is the production of β-lactamase enzyme, which breaks down the β-lactam ring and deactivates antibiotics such as penicillin, cephamycin, and carbapenem. (5, 11) Resistance through β-lactamase production has been increasingly worrisome with hundreds of different genes being specified within a single resistance mechanism.

(11) Secondly, bacteria can target the transport of drugs. An active efflux pump can move undesirable compounds, like toxic substances and antibiotics, out of cells. (12, 13) Although it exhibits natural physiological functions, the impact of it on antibiotic resistance cannot be overlooked. It accounts for most of the intrinsic resistance and more importantly the inducible aspect that can lead to higher expression of some efflux pumps upon antibiotic treatment. A third type of mechanisms involves the alterations of antibiotic intracellular target. The resistance to rifampicin, which targets RNA polymerase, can arise from a single base pair substitution in the gene that encodes RNA polymerase beta subunit (*rpoB*), which lead to the decreased affinity of rifampicin to the binding site on its target enzyme. (14)

Combat Strategies

To combat resistance, the search for novel antimicrobial agents is an indisputable approach, but the cost-effectiveness of it has been controversial. Whether is screening natural compounds that present antimicrobial activity, or targeted drug design toward inhibition of essential genes, the speed resistance can emerge to any new compound outpaces our ability to deliver new therapeutic agents. In deed, resistant organism has been isolated even before the first clinical use of antibiotics. (11) The alternative strategy is to maintain and preserve currently available antibiotics, (15) through modification of existing drugs, screening for potentiators or "co-drug" or the application of combinatorial therapy with synergistic drugs. (16)

Combinatorial Therapy

The origin of combinatorial therapy can be traced back to the 1940s, before the term was even defined, when the demonstration that penicillin and sulphonamides reinforce each other's action was made by Ungar. (17) Since then, the interactions between two or more antibiotics when used simultaneously has peaked new interests with the assumption that one type of interactions having the potential of reinforced efficacy between different drugs. In the review of Dr. Septhen D. Elek's, 1956, he indicated the many benefits of using combinatorial therapy including lowering drug resistance incidence by mathematically demonstrating the odds of a single mutant resistant to both drugs is one in a million times a million, assuming the chance of mutant resistant to each drug is one in a million. (18)

Combinatorial therapy has been suggested favor many aspects including a broader spectrum activity, different spectra of activity, lower incidence in drug resistance, beneficial usage in polymicrobial infection, and decreased toxicity of individual agents when used in synergistic combination. (19) Numerous clinical studies have been published that showed outcomes of lower mortality rate with a few examples in the table (Table 1-1) presented below.

Author	Cohort	Study design	n	Drug Combination	Outcome
Weiss et al., [8]	Pneumococcal bacteremia	Monocenter, retrospective	95	β-lactam plus macrolide	Lower mortality with combination
Dudas et al., [15]	CAP	Multicenter, prospective	2963	β-lactam plus macrolide	Lower mortality and reduced length of stay
Waterer et al., [16]	Pneumococcal bacteremia	Multicenter, retrospective	225	β-lactam plus macrolide	Lower mortality
Lodies TP et al., [17]	CAP	Multicenter, retrospective	845	β-lactam plus macrolide	Lower mortality
Rodrigo C et al., [18]	CAP	Multicenter, retropective	5240	β-lactam plus macrolide	Lower mortality

Table 1-1 Published clinical studies on combination therapy in-hospitalized patients with community-acquired pneumonia. (From (19))

With the high prevalence of antibiotic resistance and the decline in new drug development, combination therapy is the key to preserve the efficacy of current therapy, prevent new resistance, and is a rational applicable strategy that improves clinical outcome. In the following chapters, I will explore the hypersensitivity of a mutant strain, which lacks the enzyme of deoxycytidine deaminase, to specific nucleotides and a variety of antibiotics including vancomycin. Also the utilization of its phenotype to study pair-wise drug interactions of vancomycin in Gram-negative bacteria which lead to our discovery of the synergistic interaction between vancomycin and trimethoprim, that we've shown to have potent bactericidal efficacy on wild-type *E. coli.*

Chapter 1 Bibliography

1. Pena-Miller R, Laehnemann D, Jansen G, Fuentes-Hernandez A, Rosenstiel P, Schulenburg H, et al. When the Most Potent Combination of Antibiotics Selects for the Greatest Bacterial Load: The Smile-Frown Transition. *PLoS Biol. 2013;11(4): e1001540.*
2. US Department of Health and Human Services. Office of Surgeon General: Wiliam H. Steward (1965-1969). 4 Juanuary 2007. Available at http://www.surgeongeneral.gov/library/hist
3. Levy, S. B. The challenge of antibiotic resistance. 1998. *Sci. Am.* 278, 46-53.
4. CDC Office of Infectious Diseases. Antibiotic resistance threats in the United States, 2013. Available at: http://www.cdc.gov/oid/
5. Tang, S. S., Apisarnthanarak, A., Hsu, L. Y. Mechanism of β-lactam antimicrobial resistance and epidemiology of major community-and healthcare-associated multidrug-resistant bacteria. 2014. Advanced Drug Delivery Reviews. 12644.
6. Levy, S. B. Microbial resistance to antibiotics. An evolving and persistent problem. 1982. Lancet 2, 83-88
7. Levy, S. The antibiotic paradox: How misuse of antibiotics destroys their curative powers. 2002. Perseus Cambridge.
8. Wang, H., Dzink-Fox, J. L., Chen, M. and Levy, S. B. Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of acrR mutations. 2001. Antimicrob. Agents. Chemother. 45, 1515-1521

9. Schneiders, T., Amyes, S. G. B and Levy, S. B. Role of AcrR and RamA in fluoroquinolone resistance in clinical *Klebiella pneumonia* isolates from Singapore. Antimicrob. Agents Chemother. 2003. 47, 2831-2837
10. Levy, S. B., Miller, R. V. Gene transfer in the environment. 1989. McGraw Hill, New York.
11. Levy, S. B., Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. 2004. Nature Medicine Supplement. 10:12
12. Levy, S. B. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. 1992. 36, 695-703
13. Nikaido, H. Multidrug efflux pumps of gram-negative bacteria. 1996. J. Bacteriol. 178, 5853-5859
14. Garibyan, L, Huang, T, Kim, M, Wolff E, Nguyen A, Nguyen T, Diep A, Hu, K, Iverson, A, Yang, H, Miller JH. Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Esherichia coli* chromosome. 2003. DNA Repair. 13;2(5):593-608.
15. Fischbach, M. A. Combination therapies for combating antimicrobial resistance. 2011. Curr Opin Microbiol. October; 14(5): 519-523.
16. Cottarel, G., and Wierzbowski, J. 2007. Combination drugs, an emerging option for antibacterial therapy. Trends in Biotech. 25:547-555.
17. Ungar, J. Nature. 1943. 152, 245
18. Elek, S. D. Principles and problems of combined antibiotic therapy. 1956. Postgrad Med J. 32(369):324-327.

19. Mehta, K. C., Dargad, R. R., Borade, D. M., Swami, O. C. Infectious diseases: role of antibiotic combination therapy. 2014. Journal of Clinical and Diagnostic Research. Vol-8(6): ME05-ME08.

CHAPTER 2: Deoxycytidine Deaminase-deficient *Escherichia coli* Strains Display Acute Sensitivity to Cytidine, Adenosine, and Guanosine, and Increased Sensitivity to a Range of Antibiotics, Including Vancomycin

SUMMARY

We show here that deoxycytidine deaminase (DCD)-deficient mutants of *Escherichia coli* are hypersensitive to killing by exogenous cytidine, adenosine, or guanosine, whereas wild-type cells are not. The hypersensitivity is reversed by exogenous thymidine. The mechanism likely involves the allosteric regulation of ribonucleotide reductase and severe limitations of the dTTP pools, resulting in thymineless death. We also report that DCD-deficient mutants of *E. coli* are more sensitive to a series of different antibiotics, including vancomycin, and show synergistic killing between vancomycin and cytidine. One possibility is that a very low, subinhibitory amount of vancomycin gets into Gram-negative cells, and that this concentration is potentiated by chromosomal lesions resulting from the thymineless state. A second possibility is that the metabolic imbalance resulting from DCD-deficiency affects assembly of the outer membrane that normally presents a barrier to drugs such as vancomycin. We consider these findings with regard to ideas of rendering Gram negative bacteria sensitive to drugs such as vancomycin.

INTRODUCTION

A crucial step in the biosynthesis of thymidine, one of the components of DNA, is the deamination of one of the forms of deoxycytidine by the enzyme deoxycytidine deaminase, or DCD (1-5). In *Escherichia coli* and certain other Gram-negative bacteria this enzyme operates at the triphosphate level (on dCTP), deaminating dCTP to dUTP, which is then hydrolyzed to dUMP, as shown in Figure 2-1A (*e.g.*4, 5), whereas in most Gram-positive microorganisms, and in eukaryotes, this enzyme operates at the monophosphate level deaminating dCMP to dUMP (Figure 2-1B; 6,7; see 8). In both cases, the key intermediate is dUMP, since this is converted to dTMP by thymidylate syntase. Figure 2-1 also depicts a second route to the production of thymidine, namely from the conversion of UDP to dUDP by ribonucleotide reductase (RNR). The dUDP is either converted to dUTP and subsequently to dUMP, or to dUMP directly, depending on the organism (Figure 1-1A or Figure 1-1B). The UDP pools are generated either by *de novo* synthesis, or from the degradation of RNA by polynucleotide phosphorylase (PNP; 9,10). Mutants lacking DCD have decreased levels of dTTP, and increased levels of dCTP (8,11-14), leading to increased mutation rates (8,11,13-15), and in yeast to greater sensitivity to some oxidative damage (8). A third pathway exists that can also provide dUMP (16-18), involving the deamination of dC to dU by cytidine deaminase (CDD). The dU is then kinased to dUMP. This pathway can apparently alleviate the thymidine limitation in DCD-deficient mammalian cell cultures (19), as DCD-deficient mutants are thymidine requiring only if they are also deficient for CDD (12). However, in *E. coli* the *deoA* gene encoded thymidine phosphorylase degrades dU, preventing this

pathway from supplying thymidine via dUMP generation (16, and ref. therein). Inactivation of the *deoA* gene is required to enable this third pathway (16).

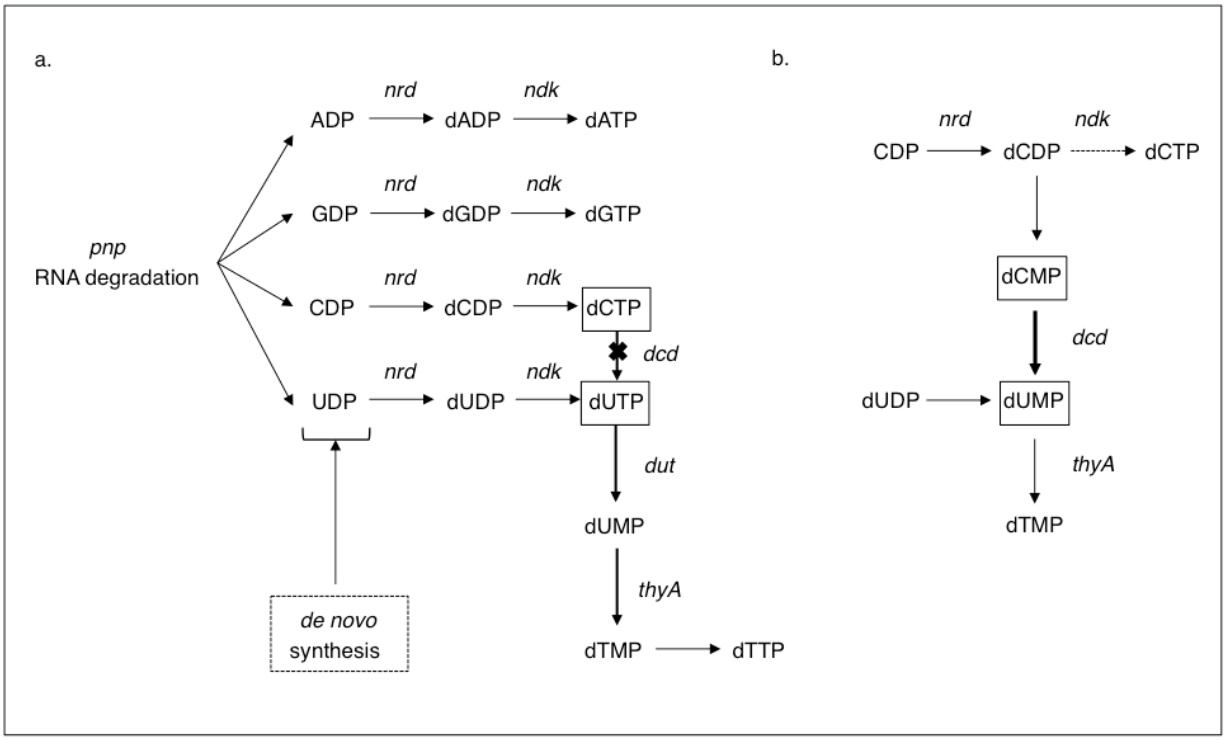

FIG 2-1 Metabolic pathway for thymidine production. a. Thymidine synthesis in *Escherichia coli*. b. Thymidine synthesis pathways in eukaryotes and Gram-positive bacteria. Note that In *Escherichia coli* the *dcd*-encoded deoxycytidine deaminase enzyme operates at the triphosphate level (on dCTP), whereas in most Gram-positive microorganisms, and in eukaryotes, this enzyme operates at the monophosphate level.

In this chapter, we show that DCD-deficient mutants of *E. coli* are hypersensitive to killing by exogenous cytidine, adenosine, or guanosine, and that this hypersensitivity is reversed by exogenous thymidine. This is in contradistinction to earlier work with different mammalian cell lines that demonstrates that these lines can be sensitive to deoxyadenosine, deoxyguanosine, and thymidine, and this sensitivity can be relieved by exogenous cytidine or deoxycytidine (20). We discuss these distinctions in light of allosteric regulation of ribonucleotide reductase. We also report here that DCD-deficient mutants of *E. coli* are more sensitive to a series

of different antibiotics, including vancomycin, and consider possible mechanisms for this increased susceptibility.

MATERIALS AND METHODS

***E. coli* strains**. The DCD-deficient strain used here is from the Keio collection, described in Baba *et al*. (21), made from the starting strain BW25113 (22). This starting strain (*lacI*^{q} *rrnB*$_{T14}$ *ΔlacZ*$_{WJ16}$ *hsdR514 ΔaraBAD*$_{AH33}$ *ΔrhaBAD*$_{LD78}$) is used as WT in the experiments reported here, unless otherwise stated. The *dcd* mutant carries a complete deletion of the *dcd* gene, with a *kan* insert in place of the gene. Strain CAG12099 (23) is MG1655 *zee-3129*::Tn*10*.

Media. The following media (24) were used. LB (10 g tryptone, 5 g yeast extract, 10 gm NaCl per liter), minimal (minimal A; 10.5g K_2HPO_4, 4.5 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate·$2H_2O$). Minimal A is the buffer used for dilutions. For growth media, minimal A is supplemented with 10ml of 20% glucose, 1 ml of 1 M $MgSO_4$, and 0.5ml of 1% thiamin hydrochloride (vitamin B1), per liter. Tryptone broth contains 10 g tryptone (1%) and 8 g NaCl per liter (24). The minimal medium A used here, when supplemented with tryptone, contains 1 g tryptone per liter (0.1%).

***E. coli* genetic methods.** Unless otherwise stated, all genetic methods are as described by Miller (24). The *dcd* mutant was purified from single colonies from the KEIO collection copy. Experiments were started by inoculation from a fresh single

colony of the *dcd* mutant. For experiments in LB, both wild-type and the *dcd* mutant were grown from single colonies during the day as a seed culture in LB supplemented with 50 μg/ml thymidine to a density of 2-3 x 10^8 cells/ml.

Determination of antibiotic sensitivity. Overnight cultures containing different concentrations of a given antibiotic were seeded with approximately 10^3 cells by inoculating 2 ml cultures with 50 μl of a 10^{-4} dilution of the over-day culture. After 18 hours incubation at 37°C on a rotor at 50 rpm, the OD_{600} was measured. Graphs of these data display percent growth versus that in LB.

Drug interaction assay. Cell cultures were prepared with the same method used in the determination of antibiotic sensitivity; using LB media supplemented with an antibiotic (vancomycin) and cytidine (see also 25) at sub-inhibitory concentrations. For experiments in minimal medium, the over-day culture in LB was spun down, washed, and resuspended in minimal A buffer before diluting 10^{-4} and seeding overnight cultures.

Time kill assay. An overnight culture of *dcd* mutant grown in minimal medium supplemented with 0.1% tryptone and 50 μg/ml thymidine was spun down, washed, and resuspended in minimal buffer. From this suspension, approximately 10^3 cells were inoculated into media containing 25 μg/ml vancomycin, 5 μg/ml cytidine, the two combined, and a control with minimal media only. Aliquots of the culture were removed at six time intervals (0, 1, 2, 3, 4, 5, and 6 hour) of incubation on a rotor with 50 rpm, and dilutions were prepared as needed for

tittering. The numbers of viable cells were determined on LB plate after 24 h incubation at 37°C with colony counts. The assay was performed in triplicate cultures and was repeated for at least twice, with representation of ± standard deviation.

Validation controls. We crossed out the *kan/dcd* insertion/replacement by P1 transduction of a linked Tn*10* marker from strain CAG12099 (23). This restored the wild-type phenotype with respect to colony size and non-sensitivity to exogenous cytidine. We then transduced the kan/dcd from the KEIO strain into the starting strain, BW25113, using the linked Tn*10* marker from, and screening the Tet^r colonies for Kan^r. The Kan^r colonies now had smaller colony size, poor growth in the absence of thymidine, and were sensitive to exogenous cytidine.

Chemicals. Kanamycin, tetracycline, chloramphenicol, rifampicin, vancomycin, nitrofurantoin, ciprofloxacin, cephradine, erythromycin, adenosine, guanosine, uridine, thymidine, and cytidine were purchased from Sigma (St. Louis, MO).

RESULTS

Cytidine, adenosine, and guanosine sensitivity of DCD-deficient mutants in LB medium. We found that *dcd* strains were strikingly sensitive to cytidine in LB medium, and also sensitive to adenosine and guanosine at higher concentrations, and that these sensitivities can be reversed by the addition of thymidine. Figure 2-

2 exemplifies this, showing overnight cultures inoculated with approximately 1,000 cells in broth medium (LB; 24; see Materials and Methods for further details) containing either no additional supplements, 100 μg/ml cytidine, or 100 μg/ml cytidine plus 50 μg/ml thymidine. (DCD-deficient mutants grow to somewhat less than full saturation in LB not supplemented with additional thymidine; see below).

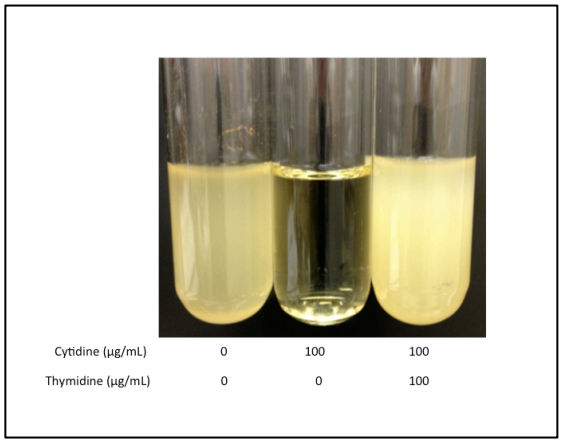

TABLE 2-1 Growth effect of cytidine on wild-type and *dcd* mutant in LB medium

Cytidine Concentration (μg/mL)	% Growth	
	WT	*dcd*
0	100	100
10	94.4 ± 8.0	2.8 ±1.0[a]
20	99.6 ± 7.4	0.0 ± 0.0
50	99.7 ± 4.1	0.0 ± 0.0

[a] Standard deviation

FIG 2-2 Growth inhibition of cytidine in *dcd* mutant and the rescue of killing by thymidine. *dcd* mutant was grown in LB medium, and with the exogenous addition of cytidine, and cytidine with thymidine, in the concentrations shown. Approximately 1,000 cells were inoculated and growth was monitored after 18 hours.

Concentrations of 20 μg/ml cytidine prevent any detectable growth, and concentrations as low as 10 μg/ml result in 97% growth inhibition (Table 2-1). In contrast, wild-type *E. coli* can tolerate concentrations of 2,000 μg/ml (Table 2-2) without evident detrimental effects on growth. In fact, higher concentrations of cytidine give enhanced growth to wild-type strains. (Higher concentrations of thymidine, or uridine also give enhanced growth to both wild-type and DCD-deificent strains, with the latter responding somewhat more to uridine than the wild-type). DCD-deficient strains are also sensitive to exogenous adenosine and guanosine (Table 2-2), although in LB higher concentrations are needed to produce

the same effect as for cytidine. For adenosine, 100 μg/ml is required for 90% inhibition of growth, and 200 μg/ml for 99% inhibition, and for guanosine 200 μg/ml are required for 83% inhibition, and 500 μg/ml for 97% inhibition. The rescue by thymidine of cytidine inhibition occurs with as little as 0.15 – 0.25 μg/ml thymidine (Figure 2-3). The inhibition/cell killing by adenosine or guanosine is also reversed by exogenous thymidine (data not shown).

TABLE 2-2 Growth effects of nucleosides on wild-type and *dcd* mutant in LB medium

Pyrimidine Concentration (μg/mL)	OD 600nm	
	WT	*dcd*
Thymidine		
0	5.50 ± 0.30	4.51 ± 0.20
100	5.56 ± 0.24	5.75 ± 0.06
1000	6.38 ± 0.19	6.45 ± 0.19
2000	7.17 ± 0.19	6.97 ± 0.11
Cytidine		
0	5.50 ± 0.30	4.51 ± 0.20
100	5.92 ± 0.24	0.00 ± 0.00
1000	6.28 ± 0.16	-
2000	6.65 ± 0.11	
Uridine		
0	5.17 ± 0.44	3.85 ± 0.21
100	5.13 ± 0.18	4.88 ± 0.40
1000	5.49 ± 0.17	6.61 ± 0.17
2000	6.16 ± 0.23	7.50 ± 0.16

Purine Concentration (μg/mL)	OD 600nm	
	WT	*dcd*
Adenosine		
0	4.92 ± 0.21	4.01 ± 0.32
100	4.97 ± 0.13	0.43 ± 0.21
200	4.91 ± 0.12	0.04 ± 0.00
Guanosine		
0	4.92 ± 0.21	4.01 ± 0.32
100	4.88 ± 0.09	1.74 ± 0.41
200	4.81 ± 0.43	0.67 ± 0.39
500	4.74 ± 0.01	0.12 ± 0.02

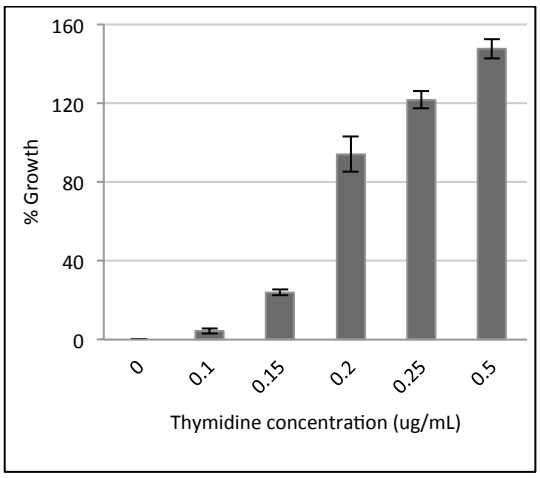

FIG 2-3 Thymidine rescues cytidine killing in *dcd* mutant. The *dcd* mutant was grown in LB medium with 50 μg/mL of cytidine and with various amount of thymidine (μg/ml). % Growth is measured against a control in LB without added cytidine (or thymidine).

Sensitivity of DCD-deficient mutants in minimal medium. In minimal medium (see Materials and Methods) supplemented with 0.1% tryptone, DCD-deficient mutants grow poorly, but respond to very low levels of thymidine for improved growth. Concentrations of 0.0025 and 0.005 μg/ml thymidine (10 nM and 20 nM, respectively) restore some growth as judged by saturation levels after 18 hours, and levels of 0.05 μg/ml restore levels near 80% of that of 50 μg/ml (Figure 2-4). Growth curves based on viable cell counts (data not shown; see Materials and Methods) validate these results, as the growth rates in 0.05 μg/ml thymidine are indistinguishable from those of 50 μg/ml (doubling time of 25 min), while the growth curves for no added thymidine are significantly lower (doubling time 44 min). Cytidine concentrations as low as 1 μg/ml prevent 95% growth (Table 2-3). This is easier to see when 0.005 μg/ml thymidine is added, as the growth levels before the addition of cytidine are higher. While 0.005 μg/ml thymidine is sufficient to restore close to 50% or higher growth levels under the conditions of the experiments in the absence of cytidine, higher concentrations (0.15 – 0.25) μg/ml

thymidine) are required to rescue the cells from cytidine-induced killing (*e.g.* Figure 2-3). Table 2-4 shows that adenosine and guanosine have roughly similar potencies to that of cytidine in minimal medium (with 0.1% tryptone broth), even though in LB neither nucleoside is as potent an inhibitor as cytidine (Table 2-2). Adenosine is slightly more potent than cytidine in this regard, and guanosine moderately less potent.

TABLE 2-3 Growth inhibition of cytidine in minimal medium with added thymidine on wild-type and *dcd* mutant

Nucleoside concentration (μg/mL)		% Growth
Cytidine	Thymidine	*dcd* mutant
0	0.005	80.1 ± 3.7
0.5	0.005	5.1 ± 0.7
1	0.005	3.8 ± 0.5
3	0.005	3.5 ± 0.4
0	50	100

TABLE 2-4 Growth effect of purines on wild-type and *dcd* mutant in minimal medium with the addition of thymidine[a]

Purine Concentration (μg/mL)	% Growth			
	Adenosine		Guanosine	
	WT	*dcd*	WT	*dcd*
0	100	100	100	100
0.1	–	98.2 ± 9.8	–	–
0.5	–	4.0 ± 0.4	–	–
1	–	2.5 ± 1.0	–	17.9 ± 1.9
5	95.6 ± 1.3	4.5 ± 1.3	–	–
10	91.6 ± 3.5	2.5 ± 0.5	83.6 ± 4.2	3.3 ± 0.7
50	–	0.6 ± 0.7	94.5 ± 3.8	2.1 ± 1.0

[a] A total of 0.005 μg/mL of thymidine was added to the medium in order to supplement growth.

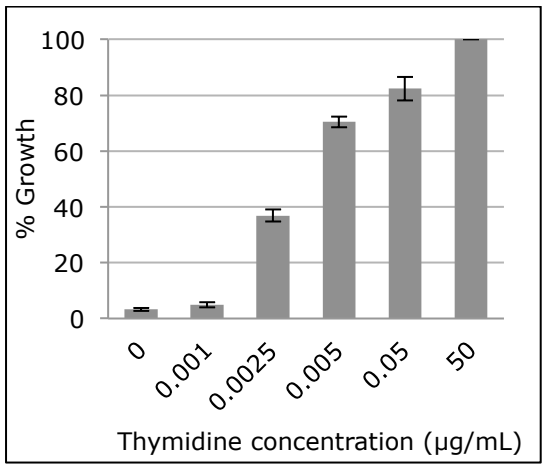

FIG 2-4 The growth potentiation by thymidine on *dcd* mutant in minimal medium. Growth restoration of different low concentrations of thymidine after 18 hours growth (see Materials and Methods), measured as % growth of the culture with 50 µg/ml thymidine.

Cytidine addition to DCD-deficient strains causes cell killing by thymineless death. In minimal medium with 0.1% tryptone, viable cell plots show that the addition of cytidine does result in cell death, as there is a loss of viability after two hours (Figure 2-5). This is not as severe as the rapid death that results in *thyA* strains (*e.g.* 26), but represents cell death nonetheless.

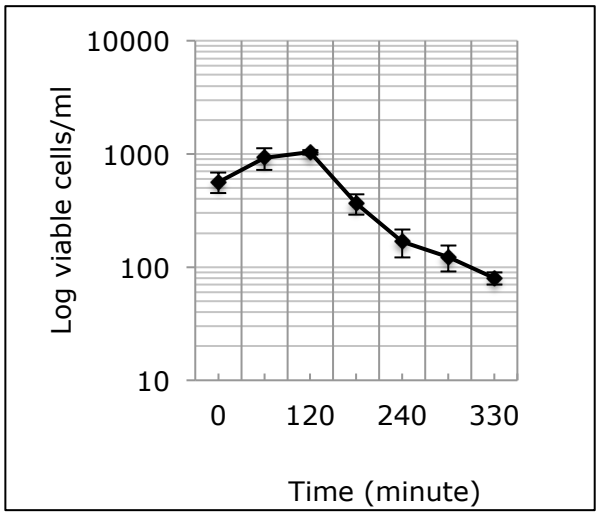

FIG 2-5 Loss of viability of *dcd* mutant in minimal medium with 50 µg/ml of cytidine. Approximately 10^3 cells were inoculated into different media (see Materials and Methods) and samples were removed at different times and directly plated onto LB medium for viable plate counts.

DCD-deficient strains show increased sensitivities to a series of antibiotics. We tested a series of antibiotics, representing different categories of antibiotics, at a range of concentrations in both the wild-type and *dcd* strain backgrounds. DCD-deficient strains are more sensitive to varying degrees to each of the antibiotics tested, ciprofloxacin (CPR), chloramphenicol (CAM), tetracycline (TET), rifampicin (RIF), cephradine (CEP), erythromycin (ERY), nitrofurantoin (NIT), and vancomycin (VAN). The concentration giving the largest difference between wild-type and the DCD-deficient derivative is displayed in Figure 2-6, and a set of concentrations for vancomycin is shown in Figure 2-7.

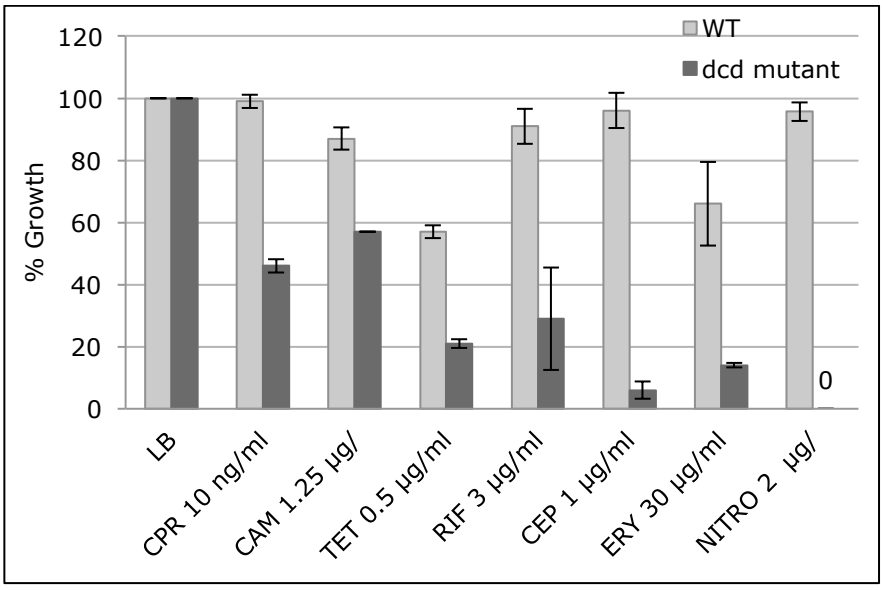

FIG 2-6 Increased sensitivity of *dcd* mutant to various antibiotics. Overnight cultures of wild-type and the *dcd* mutant containing different concentrations a given antibiotic were seeded with approximately 10^3 cells, and after 18 hours incubation at 37°C the OD_{600} was measured (see Materials and Methods). % Growth is versus the respective strain in LB with no antibiotic.

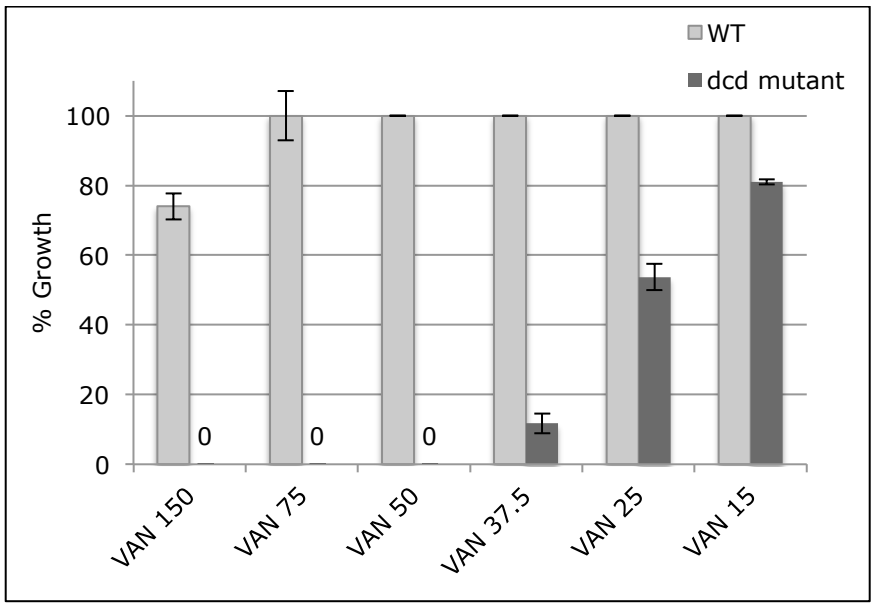

FIG 2-7 Increased sensitivity of *dcd* mutant to vancomycin (μg/ml). Cells are grown in different concentrations of vancomycin (see Legend to Figure 1-6). % growth is that compared to growth in LB alone for each individual strain.

The largest increases in sensitivity are seen for cephradine, nitrofurantoin, and vancomycin. The increased susceptibility to vancomycin is striking. Vancomycin cannot penetrate the outer membrane of Gram negative bacteria (27), resulting in a minimum inhibitory concentration (MIC of 400 μg/ml (28; data not shown). Many of the mutants that increase this sensitivity have defects in cell wall or outer membrane synthesis, although some (*e.g. recA* mutants) have defects in DNA recombination or repair functions (29,30). The fact that *dcd* mutants have an MIC for vancomycin of close to 50 μg/ml (Figure 2-7) is noteworthy, and we consider this in the discussion. Interestingly, the addition of thymidine eliminated the increased sensitivity to vancomycin (data not shown).

Cytidine-mediated killing shows synergy with vancomycin in DCD-deficient strains. Cytidine-induced killing of *dcd* strains has a synergistic effect with

vancomycin (Figure 2-8), as the combination of the two treatments gives killing significantly greater than the additive effect of each treatment alone (*e.g.* 25)**.**

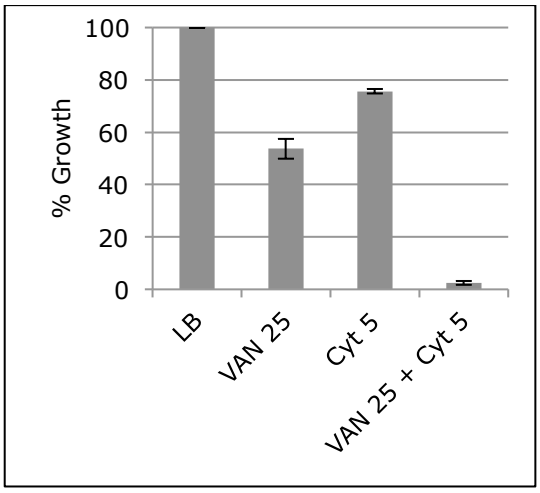

FIG 2-8 Synergistic effect of vancomycin with cytidine on *dcd* mutant. Cells are grown in LB, LB supplemented with vancomycin (25 µg/ml), cytidine (5 µg/ml), or vancomycin + cytidine). % Growth is compared to growth in LB alone.

Discussion

The size and balance of the deoxynucleotide triphosphate (dNTP) pools are important for replication fidelity (see review by Kunz et al. 31). Not only do unbalanced pools provoke an increase in mutagenesis (8,13-15,31-35), but an increase in the total pools of all four dNTPs also leads to increased mutations (36,37), and a decrease leads to reduced mutagenesis (38,39). It is not surprising, therefore, that cells have evolved intricate mechanisms to control the size and balance of dNTP pools. These control mechanisms center around ribonucleotide reductase (RNR), the essential enzyme that generates dNDP's from NDP's (see review, 40). *E. coli* has 3 different RNRs, each comprised of two different monomer subunits. The principal RNR used during aerobic growth is encoded by the *nrdA* and

nrdB genes (40, and references therein). The enzyme has a catalytic site and two separate binding sites for allosteric effectors. One effector binding site modulates the reduction of specific NDPs in response to levels of dGTP, dTTP, and dATP, and to the ratio of ATP:dATP). The second site serves as an on off switch for overall activity of the enzyme, the binding of dATP resulting in inhibition of the total activity. Here, a high ratio of dNTPs:ATP inhibits enzyme activity, where dNTP = dATP, dTTP or dGTP. (*e.g.* 40). In *E. coli*, this scheme apparently breaks down, when DCD is deficient, since the primary route to dTTP is eliminated (see Figure 2-1, and below), and the cell must rely on the UDP -> dUDP conversion by RNR to ultimately furnish dTTP (Figure 2-1A), yet RNR is now partly shut down.

DCD carries out the first step, a deamination, in converting phosphorylated derivatives of deoxycytidine ultimately to deoxythymidine (1-5; see Figure 2-1). In *E. coli*, and in other gamma proteobacteria, this step occurs at the triphosphate level (*e.g.* 4,5), whereas in most Gram-positive bacteria, and in yeast and higher cells, this step occurs at the monophosphate level (6,7). The direct effect of deleting the *dcd* gene is the buildup of dCTP pools, and the lowering of dTTP pools (5,8,11-14). As a result, this leads to increased mutagenesis in cell culture (15) yeast (8,11), and *E. coli* (13), to reduced growth rates (*e.g.* 16), and in yeast to moderately increased sensitivity to DNA damaging agents (8). The increased mutagenesis is reduced or eliminated by the addition of thymidine (15; data not shown). Here, we show that *E. coli dcd* mutants are extremely sensitive to exogenous adenosine, guanosine, or cytidine, in contradistinction to wild-type *E. coli*. The *dcd* mutants grow slowly in the absence of thymidine, because the primary pathway for thymidine synthesis involves the DCD catalyzed deamination of dCTP,

which is now blocked (see Figure 2-1A.) The second route, via RNR reduction of UDP -> dUDP provides only 20% of the normal amount of thymidine in *E. coli* (41, and references therein). Adding even very low levels of thymidine improves growth to near normal levels (Figure 2-4). However, as Tables 2-4 shows, the addition of cytidine, adenosine, or guanosine kills *dcd* cells. Thus, it would appear that exogenous cytidine, adenosine, or guanosine (rC, rA, or rG) is converted to enough dCTP, dATP, or dGTP to sufficiently shut down RNR to the point that an insufficient level of thymidine is provided via the UDP -> dUDP pathway. Moreover, Buttin and coworkers have shown that CTP strongly inhibits UDP reduction by RNR from Chinese hamster cells (12). This shutting down of RNR in a DCD-deficient background leads the cell into thymineless death (Figure 2-5), the latter being a well-studied phenomenon (*e.g.* 26,42,43). A potential third pathway, involving deamination of deoxycytidine (dC) to deoxyuracil (dU) by cytosine deaminase (CDD) is not available in *E. coli* unless the *deoA* gene is inactivated (16,18), as the *deoA*-encoded enzyme is a deoxycytidine phosphorylase that normally degrades dC and dU (18). In mammalian cell lines, this third pathway is active, so that cell lines such as human B lymphoblasts (Raji) that carry a *dcd* mutation are not sensitive to cytosine (19), as they are in *E. coli*, unless they are also deficient in CDD, as is the case with Chinese hamster cell lines (12).

Wild-type *E. coli* is not sensitive to any of the exogenous nucleosides (see *e.g.* Table 2-2) in concentrations as high as 2-4 x 10^{-3} M. In cultured cells, the expansion of the dATP, dGTP, or dTTP pools results in specific feedback inhibition of RNR (*e.g.* 40), ultimately resulting in lowered levels of dCTP (20). (This is the inverse of the findings here for d*cd* mutants, in which expansion of the other dNTP

pools limits the dTTP pool). Thus, different mammalian cell lines have been shown to be sensitive to levels of 10^{-5} M -> 10^{-4} M of exogenous adenosine, deoxyadenosine, deoxyguanosine, or thymidine (20, 44, and references therein), and this toxicity can be relieved by exogenous cytidine or deoxycytidine (20). Some mammalian cell culture mutants resistant to deoxyadenosine or deoxyguanosine were shown to have altered allosteric regulation of RNR (34,45,46), with RNR now responding less to, *e.g.* dGTP. Other resistant mutants had increased levels of RNR (47), and some mutants resistant to adenosine had hyperactive adenosine-deaminase (44), an enzyme that normally reduces the toxic level of adenosine. Certain autoimmune diseases result from the inherited lack of this enzyme (see 45).

The increased sensitivity to antibiotics (Figures 2-6, 2-7) is highlighted by the greatly increased sensitivity to vancomycin, a glycopeptide that cannot normally penetrate the outer membrane of Gram-negative bacteria (27,48), precluding its use against Gram-negative infections. It is noteworthy that vancomycin acts synergistically with cytidine (Figure 2-8). The increased sensitivity to vancomycin is eliminated by the addition of thymidine (data not shown). How can the excess of dCTP and the decrease in the dTTP pools result in increased sensitivity to vancomycin? One possibility is that in the partial thymineless state, or (in the presence of exogenous cytidine) full thymineless state, there is a buildup of irreparable chromosomal lesions (43) that act synergistically by potentiating a very small and subinhibitory concentration of vancomycin that does get into the cell and that itself results in DNA damage. Collins and coworkers have proposed and presented evidence for the idea that all bactericidal antibiotics generate hydroxyl

radicals that kill the cell by causing DNA damage (49), and that the oxidation of guanine to 8-oxodGuanine in the nucleotide pools is a key mechanism by which this occurs (50). An expanded examination of synergistic effects of vancomycin and other antibiotics in a DCD-deficient background might shed light on this model. A second possibility is that the excess of dCTP or the decrease in the dTTP pools in a DCD-deficient mutant triggers a metabolic imbalance that in some way affects outer membrane assembly. The phenotype of DCD-deficient mutants with respect to antibiotics is similar to, although less severe than, that seen for SurA-deficient mutants that are greatly affected in outer membrane assembly. SurA is a crucial folding factor/chaperone for beta-barrel proteins (29). If outer membrane assembly is aberrant, then proteins such as OmpA, OmpF, LamB, OmpC, and LptD are incorrectly folded and rapidly degraded, which can be tested with antibodies to these proteins using Western Blots. Interestingly, a survey of the KEIO collection of gene knockouts in *E. coli* found that of the few mutants showing increased susceptibility to vancomycin, some involved defects in DNA repair or recombination (e.*g. recA* mutants), and others in outer membrane synthesis (28,30).

Combinatorial strategies for antibiotic use offer an expanded repertoire of drug therapies (51). Of particular interest are potentiators of existing antibiotics, or "codrugs", a number of which have been used in both laboratory (52,53), and clinical (54-56) settings. Also, Collins and coworkers have demonstrated that specific metabolites can potentiate aminoglycosides acting on *E. coli* and *Staphylociccus aureus* biofilms (57). One desired potentiator would sensitize Gram-negative bacteria to vancomycin. The *dcd* mutants described here have a MIC of 50 μg/ml for vancomycin, suggesting that small molecule inhibitors of DCD would have

the same effect and would be useful potentiators. The 3D structure of *E. coli* DCD is known (41,58), facilitating drug design. The *E. coli* DCD enzyme shares extensive homology among Gram-negative bacteria. Figure 2-9 compares the DCD sequence among a set of enzymes from different gamma protebacteria that are pathogens. The *E. coli* enzyme is 96% identical to individual members of this group, such as *Klebsiella pneumoniae*, with the group as a whole sharing 80% identity. It appears likely that an inhibitor of the *E. coli* enzyme would also inhibit, for instance, the DCD enzyme from *K. pneumoniae*, the leading cause of nosocomial infection among patients in intensive care units (59). Such a potentiator would have a narrow spectrum of action, since DCD from Gram-positive bacteria and from humans has a different substrate (dCMP instead of dCTP), and the enzymes from *Bacillus subtilis* and from humans share only weak homology (25% and 26% identity, respectively) to the *E. coli* enzyme. It is highly unlikely that an inhibitor of the *E. coli* enzyme would affect either the Gram-positive population or human cells. It is not clear how many other Gram negative bacteria outside of the gamma proteobacteria use DCD at the triphosphate level and how many use DCD at the monophosphate level, since an examination of the homologies to each of these enzymes from members of different classes of Gram-negative bacteria give ambiguous results (data not shown). Biochemical experiments are needed to answer this question. Other mutants shown to increase the sensitivity of *E. coli* to vancomycin (s*urA*, 29; *smpA*, 28) suggest targets as well. Also, recently, Collins and coworkers have shown that silver sensitizes *E. coli* to a series of antibiotics, including vancomycin (60), and Wink and coworkers have reported that EDTA potentiates the combination of vancomycin plus thymidine (61).

FIG 2-9 Homologous alignment of DCD among

CHAPTER 2 Bibliography

1. McMurry, J.E., and Begley, T. P. 2005. The Organic Chemistry of Biological Pathways, Roberts and Company, Englewood, N. J.
2. Neuhard, J., and Kelin, R. 1996. Biosynthesis and conversions of pyrimidines, p. 580-599. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.) *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D. C.
3. Neuhard, J., and Thomassen, E. 1976. Altered deoxyribonucleotide pools in P2 eductants of *Escherichia coli* K-12 due to a deletion of the *dcd* gene. J. Bacteriol. 126:999-1,001.
4. O'Donovan, G. A., and Neuhard, J. 1970. Pyrimidine metabolism in microorganisms. 34:278-343.
5. O'Donovan, G. A., Edlin, G., Fuchs, J. A., Neuhard, J., and Thomassen, E. 1971. Deoxycytidine triphosphate deaminase: Characterization of an Escherichia coli mutant deficient in the enzyme. J. Bacteriol. 105:666-672.
6. Ellims, P. H., A. Kao, A. Y., and Chabner, B. A. 1981. Deoxycytidylate deaminase. Purification and some properties of the enzyme isolated from human spleen. J. Biol. Chem. 256:6335-6340.
7. McIntosh E. M., and Haynes, R. H. 1984. Isolation of a *Saccharomyces cerivasiae* mutant strain deficient in deoxycytidine deaminase activity and partial characterization of the enzyme. J. Bacteriol. 158:644-649.

8. Sanchez, A., Sharma, S., Rozenzhak, S., Roguev, A., Krogan N. J., Chabes, A., and Russell, P. 2012. Replication fork collapse and genome instability in a deoxycytidylate deaminase mutant. Mol. Cell. Bio. 32:4445-4454.
9. Danchin, A. 1997. Comparison between the *Escherichia coli* and *Bacillus subtilis* genomes suggests that a major function of polynucleotide phosphorylase is to synthesize CDP. DNA Res. 4:9-18.
10. Donovan, W. P., and Kushner, S. R. 1986. Polynucleotide phosphorylase and ribonuclease II are required for cell viability and mRNA turnover in Escherichia coli K-12. Proc. Natl. Acad. Sci. USA 83:120-124.
11. Kohalmi S. E., Glattke, M., McIntosh, E.M., and Kunz, B. A. 1991. Mutational specificity of DNA precursor pool imbalances in yeast arising from deoxycytidylate deaminase deficiency or treatment with thymidylate. Mutat. J. Mol. Biol. 220:933-946.
12. Robert de Saint Vincent, B., Dechamps, M., and Buttin, G. 1980. The modulation of the thymidine triphosphate pool of Chinese hamster cells by dCMP deaminase and UDP reductase. J. Biol. Chem. 233:162-167.
13. Schaaper, R., and Mathews, C. K. 2013. Mutational consequences of dNTP pool imbalances in *E. coli*. DNA Repair 12:73-79.
14. Weinberg, G. L., Ullman, B., and Martin, D. W. Jr. 1981. Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proc. Natl. Acad. Sci. USA. 78:2447-2451.

15. Weinberg, G. L., Ullman, B., Wright, C. M., and Martin, D. W. Jr. 1985. The effects of exogenous thymidine on endogenous deoxynucleotides and mutagenesis in mammalian cells. Somatic Cell and Mol. Gen. 11:413-419.
16. Weiss, B. 2007. The deoxycytidine pathway for thymidylate synthesis in *Escherichia coli*. J. Bacteriol. 189:7922-7926.
17. Weiss, B. 2007. YjjG, a dUMP phosphatase, is critical for thymine utilization by *Escherichia coli* K-12. J. Bacteriol. 189:2186-2189.
18. Weiss, B. and Wang, L. 1994. De novo synthesis of thymidylate via deoxycytidine in *dcd* (dCTP deaminase) mutants of *Escherichia coli*. J. Bacteriol. 176:2194-2199.
19. Perignon, J.-L., Thullier, L., Hamet, M., Houlier, A.-M., and Cartier, P.-H. 1986. Synergistic toxicity of pyrazofurin and cytidine in cytidine deaminase deficient lymphoid cells (Raji). Int. J. Immunopharmac. 8:427-431.
20. Dahbo, Y., and Eriksson, S. 1985. On the mechanism of deoxyribonucleoside toxicity in human T-lymphoblastoid cells. Eur. J. Biochem. 150:429-434.
21. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., M. Tomita, B. L. Wanner, B. L., and Mori, H. 2006. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: The Keio collection. Mol. Sys. Biol. 2:2006.0008.
22. Datsenko, K. A., and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97:6640-6645.
23. Singer, M., Baker, T. A., Schnitzler, G., Deischel, S. M., Goel, M., Dove, W., Jaacks, K. W., Grossman, A. D., Erickson, J. W., and Gross, C. A. 1989. A

collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbial. Rev. 53:1-24.

24. Miller, J. H. 1992. A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia coli* and related bacteria, p. 194-195. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. Yeh, P., Tschumi, A. I., and Kishony, R. 2006. Functional classification of drugs by properties of their pairwise interactions. Nature Genetics. 28:489-494.
26. Ohkawa, T. 1975. Studies of intracellular thymidine nucleotides. Thymineless death and the recovery after re-addition of thymine in *Escherichia coli* K12. Eur. J. Biochem. 60:57-66.
27. Nikaido, H. 1989. Outermembrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. 33:1831-1836.
28. Tamae, C., Liu, A., Kim, K., Sitz,, Hong, D. J., Becket, E., Bui, A., Solaimani, P., Tran, K. P., Yang, H., and Miller, J. H. 2008. Determination of antibiotic hypersensitivity among 4,000 single gene knockout mutants of *Escherichia coli*. J. Bacteriol. 190:5981-1988.
29. Justice, S. S., Hunstad, D. A., Harper, J. R., Duguay, A. R., Pinkner, J. S., Bann, J., Frieden, C., Silhavy, T. J., and Hultgren, S. J. 2005. Periplasmic peptidyl prolyl *cis-trans* isomerases are not essential for viability, but SurA is required for pilus biogenesis in *Escherichia coli*. J. Bacteriol. 187:7680-7686.
30. Liu, A., Tran, L., Becket, E., Lee, K., Chinn, L., Parker, E., Tran, K., and Miller, J. H. 2010. Antibiotic sensitivity profiles determined with an

Escherichia coli gene knockout collection: Generating an Antibiotic bar code. Antimicrob. Agents Chemother. 54:1393-1403.

31. Kunz, B. A., Kohalmi, S. E., Kunkel, T. A., Mathews, C. K., McIntosh, E. M., and Reidy, J. A. 1994. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. Mutat. Res. 318:1–64.
32. Lu, Q., Zhang, X., Almaula, N., Mathews, C. K., and Inouye, M. 1995. The gene for nucleoside diphosphate kinase functions as a mutator gene in *Escherichia coli*. J. Mol. Biol. 254:337–341.
33. Meuth, M. 1989. The molecular basis of mutations induced by deoxyribonucleoside triphosphate pool imbalances in mammalian cells. Exp. Cell Res. 181:305-316.
34. Meuth, M., Aufreiter, E., and Reichard, P. 1976. Deoxyribonucleotide pools in mouse- fibroblast cell lines with altered ribonucleotide reductase. Eur. J. Biochem. 71:39-43.
35. Miller, J.H., Funchain, P., Clendenin, W., Huang, T., Nguyen, A., Wolff, E., Yeung, A., Chiang, J., Garibyan, L., Slupska, M. M., and Yang, H. 2002. *Escherichia coli* strains (*ndk*) lacking nucleoside diphosphate kinase are powerful mutators for base substitutions and frameshifts in mismatch repair deficient strains. Genetics,162:5-13.
36. Gon, S., Napolitano, R., Rocha, W., Coulon, S., and Fuchs, R. P. 2011. Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced-mutagenesis in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 108:19311-19316.

37. Wheeler, L. J., Rajagopal, I., and Mathews, C. K. 2005. Stimulation of mutagenesis by proportional deoxynucleoside triphosphate accumulation in *Escherichia coli*. DNA Repair 4:1450-1456.

38. Becket, E., Tse, L., Yung, M., Cosico, A., and Miller, J. H. 2012. Polynucleotide phosphorylase plays an important role in the generation of spontaneous mutations in *Escherichia coli*. J. Bacteriol. 194:5613-5620.

39. Laureti, L., Selva, M., Dairou, J., and Matic, I. 2013. Reduction of dNTP levels enhances DNA replication fidelity in vivo. DNA Repair 12:300-305.

40. Hofer, A., Crona, M., Logan, D. T., and Sjöberg, B. M. 2012. DNA building blocks: keeping control of manufacture. Crit. Rev. Biochem. Mol. Biol. 47:50-63.

41. Thymark, M., Johansson, E., Larsen, S., and Willmoës, M. 2008. Mutational analysis of the nucleotide binding site of Escherichia coli dCTP deaminase. Arch. Biochem. Biophys. 470:20-26.

42. Cohen, S. S., and HBarner, H. D. 1954. Studies on unbalanced growth in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 40:885-893.

43. Kuong, K. J., and Kuzminov, A. 2010. Stalled replication fork repair and misrepair during thymineless death in *Escherichia coli*. Genes to Cells 15:619-634.

44. Fernandez-Mejia, C., Debatisse, M., and Buttin, G. 1984. Adenosine-resistant Chinese hamster fibroblast variants with hyperactive adenosine-deaminase: An analysis of the protection against exogenous adenosine afforded by increased activity of the deamination pathway. J. Cell. Physiol. 120:321-328.

45. Ullman, B., Gudas, L. J., Clift, S. M., and Martin, D. W., Jr. 1979. Isolation and characterization of purine-nucleoside phosphorylase-deficient T-lymphoma cells and secondary mutants with altered ribonucleotide reductase: Genetic model for immunodeficiency disease. Proc. Natl. Acad. Sci. USA 76:1074-1078.

46. Ullman, B., Gudas, L. J., Caras, I. W., Erilsson, S., Weinberg, G. L., Wormsted, M. A., and Martin, D. W. Jr. 1981. Demonstration of normal and mutant proteins M1 subunits of deoxyGTP-resistant ribonucleotide reductase from mutant mouse lymphoma cells. J. Biol. Chem. 256:10189-10192.

47. Meuth, M., and Green, H. 1974. Alterations leading to increased ribonucleotide reductase in cells selected for resistance to deoxynucleoside. Cell 3:367-374.

48. Pages, J. M., James, C. E., and Winterhalter, M. 2008. The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. 6:893-903.

49. Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797-810.

50. Foti, J. J., Devadoss, B., Winkler, J. A., Collins, J. J., and Walker, G. C. 2012. Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. Science 336:315-319.

51. Cottarel, G., and Wierzbowski, J. 2007. Combination drugs, an emerging option for antibacterial therapy. Trends in Biotech. 25:547-555.

52. Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., D. Cho, D., Chamberland, S., T. Renau, T., Ishida, H., and Lee, V. J. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. Antimicrob. Agents Chemother. 45:105-116.

53. Nelson, M. L., and Levy, S. B. 1999. Reversal of tetracycline resistance by different bacterial tetracycline resistance determinants by an inhibitor of the Tet(B) antiport protein. Antimicrob. Agents Chemother. 43:1719-1724.

54. Buynak, J. D. 2006. Understanding the longevity of the β-lactam antibiotics and of antibiotic/β-lactamase inhibitor combinations. Biochemical Pharmacology 71:930-940.

55. Hidaka, M., Kawashima, K., Yoshida, M., and Mitsuhashi, S. 1992. Inactivation of new carbapenem antibiotics by dehydropeptidase-1 from porcine and human renal cortex. J. Antimicrob. Chemother. 30:129-134.

56. Hoffman, J., Trimble, J., and Brophy, G. M. 2009. Safety of imipenem/cilastin in neurocritical care patients. Neurocrit. Care 10:403-407.

57. Allison, K. R., Brynildsen, M. P., and Collins, J. J. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216-220.

58. Johansson, E., Fano, M., Bynck, J. H., Neuhard, J., Larsen, S., Sigurskjold, B. W., Christensen, U., and Willemoës, M. 2005. Structures of dCTP deaminase from *Escherichia coli* with bound substrate and product: Reaction mechanism and determinants of mono- and bifunctionality for a family of enzymes. J. Biol. Chem. 280:3051-3059.

59. Lockhardt, S. R., Abramson, M. A., Beekmann, S. E., Gallagher, G., Riedel, S., Diekema, D. J., Quinn, J. P., and Doern, G. V. 2007. Antimicrobial resistance among gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004. J. Clin. Microbiol. 45:3352-3359.

60. Morones-Ramirez, J. R., Winkler, J. A., Spina, C. S., and Collins, J. J. 2013. Silver enhances antibiotic activity against Gram negative bacteria. Science Transl. Med. 5:190ra81.

61. Hamoud, R., Zimmermann, S., Reichling, J., and Wink, M. 2013. Synergistic interactions in two-drug and three drug combinations (thymol, EDTA, and vancomycin) against multi drug resistant bacteria including *E. coli*. doi: 10.1016/j. phymed.

CHAPTER 3: Identification of Synergistic Interaction between Vancomycin and Trimethoprim and Its Potency In Wild-type *Escherichia coli*.

Summary

The increased sensitivity to vancomycin (VAN) of *dcd* mutant led us to the exploration for potential synergistic antibiotics in a pair-wise combination with VAN, which is normally limited to the use on Gram-positive bacteria, due to permeation barrier from an outer membrane of Gram-negative bacteria. In this chapter, we show the study of ten different antibiotics, representing six different functional categories, of their interactions with VAN. Among those, trimethoprim (TMP) presents a striking synergy with VAN, which activates bactericidal efficacy together with VAN as low as 6.25 μg/ml on a wild-type strain. This intriguing effect exhibits a valuable combinatorial therapy of VAN and TMP on wild-type *E.coli* or other Gram-negative bacteria.

Introduction

Vancomycin (VAN) has been proved to be an effective antibiotic against certain multi-drug resistant Gram-positive pathogens, such as MRSA (methicillin-resistant *Stapylococcus aureus*). However, the large size of this glycopeptide precludes it from being useful against Gram-negative infections, since the outer membrane of Gram-negative bacteria acts as a barrier to its entry into the cell (1). Our recent study in deoxycytidine deaminase deficient (DCD) mutant, which presents an increased sensitivity to VAN, led us to the rational of the utilization of

dcd mutant to exlopre potential drug combinations with VAN. We detected strong synergies between VAN and TMP, in not only the mutant strain, but also the wild-type *E. coli* strain. This result exhibits particular value in the possible implications of new therapeutic remedy of treating resistant Gram-negative bacteria, such as carbapenem-resistant *Klebsiella pneumoniae*, a leading cause of hospital-acquired pneumonia in the US (2).

Materials and Methods

***E. coli* strains**. The DCD-deficient strain used here is from the Keio collection, described in Baba *et al*. (3), made from the starting strain BW25113 (4). This starting strain ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) is used as wild-type (WT) in the experiments reported here, unless otherwise stated. BW25113, as closely related to MG1655, as both are derived from the strain background W1485 (3). The *dcd* mutant carries a complete deletion of the *dcd* gene, with a *kan* insert in place of the gene.

Media. The following media (5) were used. LB (10 g tryptone, 5 g yeast extract, 10 gm NaCl per liter).

***E. coli* genetic methods.** Unless otherwise stated, all genetic methods are as described by Miller (5). The *dcd* mutant was purified from single colonies from the KEIO collection copy. Experiments were started by inoculating from a culture made from a single colony and stored in glycerol (5) at -80°C. A sample of the frozen

glycerol culture was inoculated into LB medium and grown for 6 hours in a water bath at 37º before being used to seed overnight cultures with approximately 10^3 cells. This was achieved by inoculating 2 ml cultures with 50 μl of a 10^{-4} dilution of the over-day culture. After 18 hours incubation at 37°C on a rotor at 50 rpm, the OD_{600} was measured. Graphs of these data display percent growth versus that in LB.

Determination of single-drug concentrations. Ten different antibiotics were selected as representations of six functional groups in terms of their killing mechanisms, as previously defined by Yeh, Tchumi and Kishony (6). Overnight cultures containing a range of concentrations of a given antibiotic (usually from the reported MIC with twofold intervals) were seeded with approximately 10^3 cells by inoculating 2 ml cultures with 50 μl of a 10^{-4} dilution of the over-day culture. After 18 hours incubation at 37°C on a rotor at 50 rpm, the OD_{600} was measured. Sub-inhibitory concentrations were chosen at typically 60% to 80% reduction in growth compared to the growth in controls with only LB. For the experiment of variable aeration and cell density, the inoculation was made with 50 μl of a 10^{-4}, a 10^{-3}, a 10^{-2}, and a 10^{-1} dilution of the over-day culture; followed by 18 hours incubation at 37°C either on a rotor at 50 rpm or the incubator (without aeration).

Drug interaction assay. Cell cultures were prepared with the same method used in the determination of single drug concentration; using LB media supplemented with vancomycin and a second antibiotic at sub-inhibitory concentrations. Bar graphs were used to compare the effects of the paired drugs with those of the corresponding single drugs at the same dose, and the controlled growth in LB only.

Time kill assay. Wild-type *E. coli* cultures were inoculated with approximately $1x10^4$ CFU/ml, from a resuspension in MC buffer stored at 4°C, into LB media containing 100 μg/ml of vancomycin, 0.15 μg/ml of trimethoprim, the two drugs combined, and a control with LB media only. Aliquots of the culture were removed at five time intervals (0, 2, 4, 6, and 8 hour) of incubation on a rotor with 50 rpm, and dilutions were prepared as needed for tittering. The numbers of viable cells were determined on LB plate after 24 h incubation at 37°C with colony counts. The assay was performed in triplicate cultures and was repeated for at least twice, with representation of ± standard deviation.

Classification of Drug Interactions. Additivity is defined as $W_{xy} = W_x W_y$ where W_x is the growth rate of drug X , W_y is the growth rate of drug Y, and W_{xy} is the growth rate of the drugs combined. Deviation from addivitity is defined by $\tilde{\varepsilon}$, which is calculated from the formulas below. When $\tilde{\varepsilon}$ falls within the range of -1 to -0.5, we classify it as synergistic; when $\tilde{\varepsilon}$ is between -0.5 and 0.5, we classify it as additive; when $\tilde{\varepsilon}$ falls between 0.5 and 2, we classify it as antagonistic.

$$\tilde{\varepsilon} = {W_{xy} - W_x W_y} \Big/ {\left|\widetilde{W}_{xy} - W_x W_y\right|}$$

$\widetilde{W}_{xy}$ = min $[W_x, W_y]$ for $W_{xy} > W_x W_y$, and is 0 otherwise

If $\widetilde{W}_{xy}$ > min $[W_x, W_y]$, then

$$\tilde{\varepsilon} = \left[\left(W_{xy} - \min\left[W_x,\ W_y\right] \right) \Big/ \left(1 - \min\left[W_x,\ W_y\right]\right) \right] + 1$$

Chemicals. Kanamycin, tetracycline, chloramphenicol, rifampicin, vancomycin, nitrofurantoin, ciprofloxacin, cefoxidine, erythromycin, trimethoprim, and clindomycin were purchased from Sigma (St. Louis, MO).

Results

Pair-wise drug combinations. We initially tested a set of 10 antibiotics against a subinhibitory concentration of VAN in *dcd* mutant. Whereas the MIC (minimum inhibitory concentration) for VAN for the wild-type is 400 μg/ml (data not shown) under the conditions of the experiment (see Materials and Methods), the MIC for the *dcd* mutant is 50 μg/ml, and 25 μg/ml was used as a subinhibitory concentration.

Table 3-1 List of antibiotics used in the study with dosage and primary targets

Antibiotic	Abbreviation	Dosage (μg/ml)	Primary Target
Cefoxitin	FOX	1	Cell wall synthesis
Chloramphenicol	CHL	0.25	Protein synthesis, 50S
Ciprofloxacin	CPR	0.01	DNA gyrase
Clindamycin	CLI	8	Protein synthesis, 50S
Erythromycin	ERY	5	Protein synthesis, 50S
Nitrofurantoin	NIT	0.5	DNA
Streptomycin	STR	0.6	Protein synthesis, 30S

Tetracycline	TET	0.075	Protein synthesis, 30S
Tobramycin	TOB	2	Protein synthesis, 30S
Trimethoprim	TMP	0.15	Folic acid biosynthesis
Vancomycin	VAN	25	Cell wall synthesis

Table 3-1 lists the antibiotics used in this study, and the concentrations used in the initial tests for synergy. Figure 2-1 and displays the results in the format employed by Yeh, Kishony and coworkers (6). This latter study characterized the interactions as additive, suppressive, antagonistic, and synergistic (see Materials and Methods for a fuller explanation). Here, percent residual growth versus growth in LB without antibiotic is plotted for each single antibiotic, and for the pair of antibiotics. Synergistic effects are those that are significantly greater than simple additive effects. From Figure 3-1, we assign merely additive effects (no synergy) to the combinations of VAN with CLI, TOB, TET and STR; weak synergies with VAN and CPR, CHL ERY and FOX; and very strong synergies with VAN and either NIT or TMP.

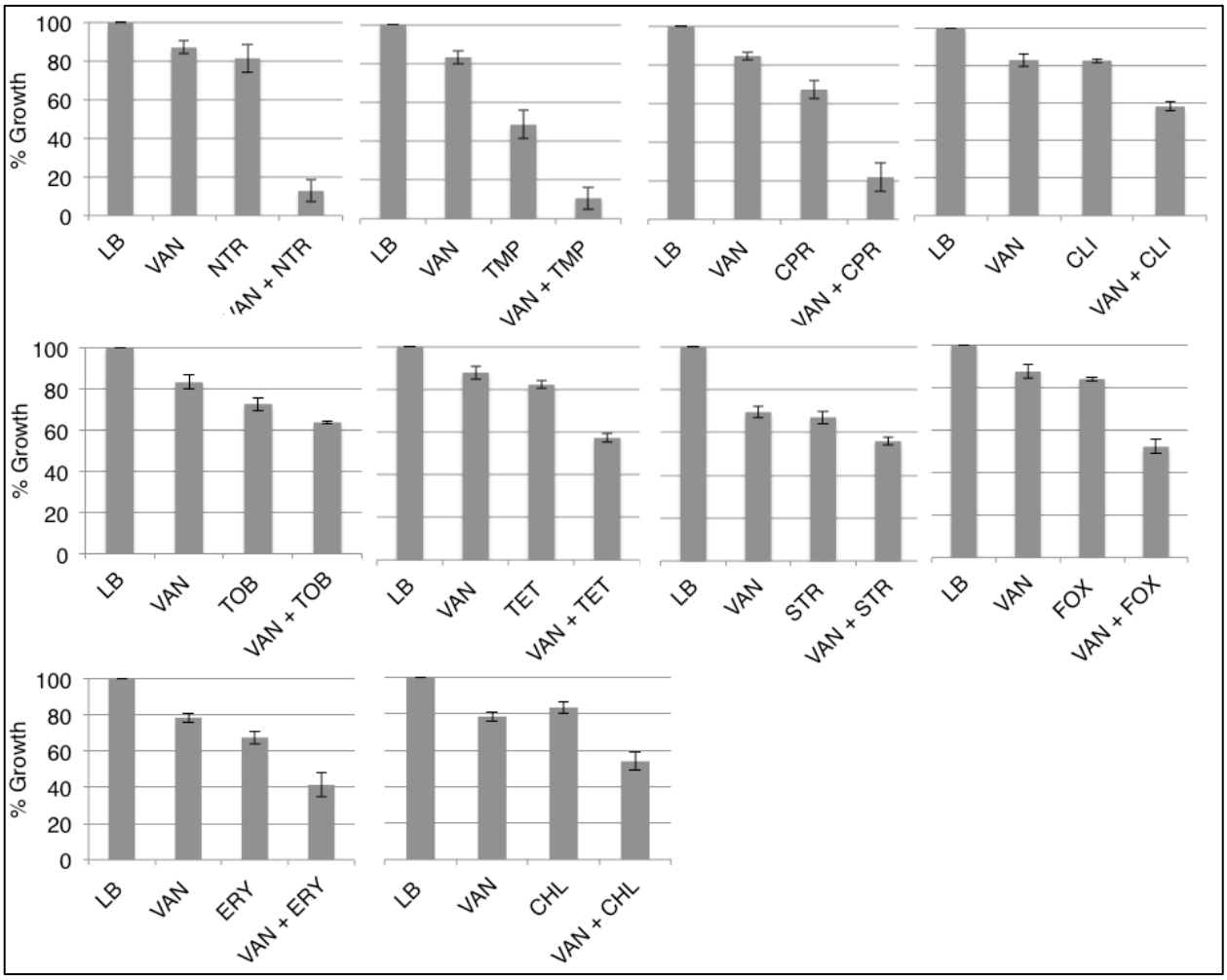

FIG 3-1. Interactions of different drug combinations with vancomycin in *dcd* mutant. Comparison graphs were plotted with bars from left to right as (LB) no drug, VAN, the second drug, and the combination of two drugs against growth percentage on the y-axis. Refer to Table 2-1 for the concentrations used.

Synergy in the wild-type background. The strength of the synergies with VAN and TMP suggests that the effect might be strong enough to be potent in the wild-type background. This is indeed the case, as Figures 3-2 demonstrates. The combination of VAN and TMP shows effects with concentrations as low as 6.25 μg/ml VAN (Figure 3-2)

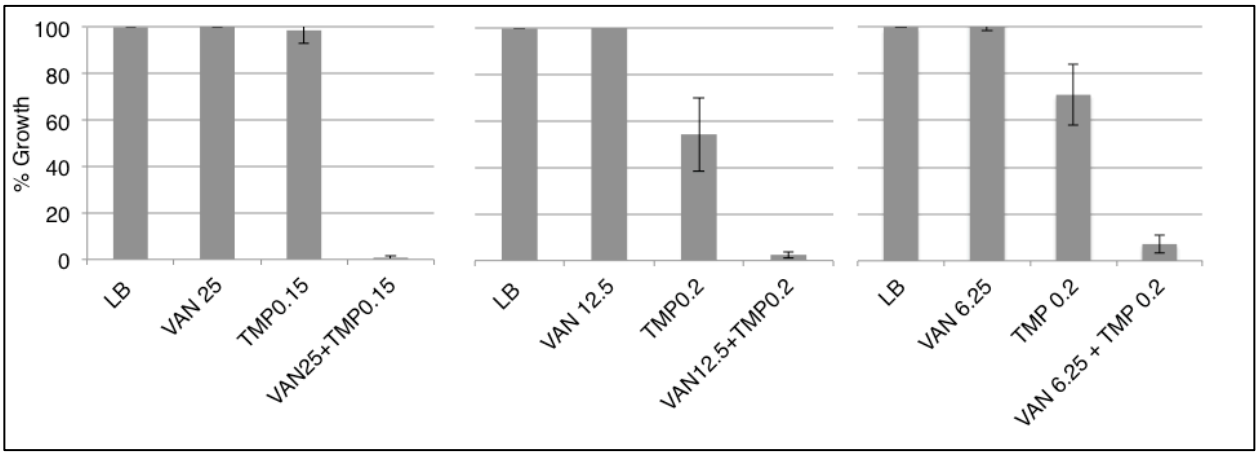

FIG 3-2. The synergistic interaction between vancomycin (μg/ml) and trimethoprim (μg/ml) in WT. *E. coli* was grown up in vancomycin, trimethoprim, and the two drugs combined. Growth percentages at each concentration were calculated against the growth in LB medium. (See Materials and Methods)

Aeration and cell density. Oxidative damage or the accumulation of free radicals was believed to contribute antimicrobial activity to certain degrees (7). Cell density can also be a limiting factor with regard to potency. Considering the experimental design of the synergy experiment, in terms of the aeration given and starting number of cells, we examined and showed that the efficacy of synergy between VAN and TMP, with the concentration reported before, was not limited or dependent on aeration or cell density. Figure 3-3 demonstrates the consistency of synergy when various inoculations were made with a range from approximately 10^3 to 10^6 cells/ml, with or without aeration (See Materials and Methods).

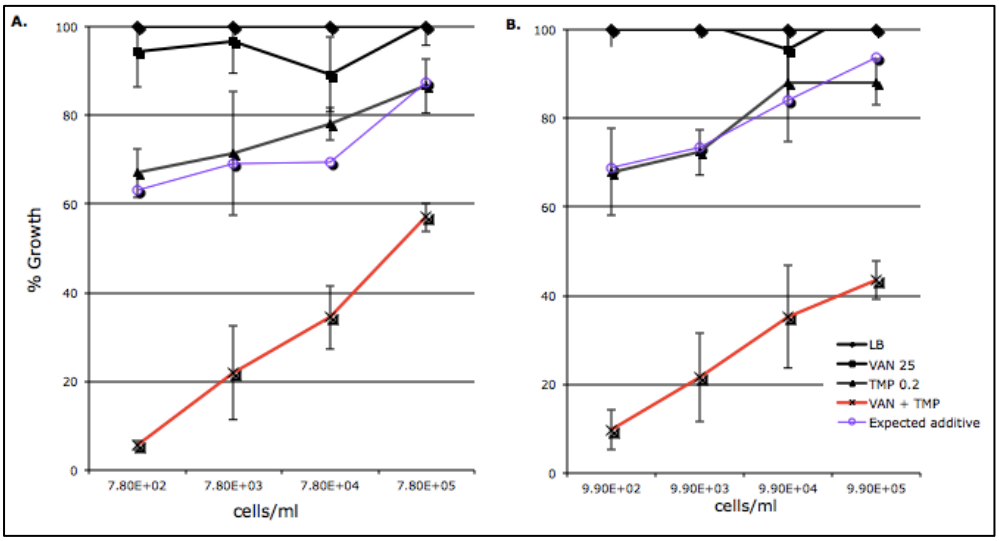

FIG 3-3. The effects of aeration and cell density on the potency of the synergy between VAN and TMP. Concentrations used here for VAN was 25 μg/ml and 0.2 μg/ml for TMP. (A.) The experiment was conducted with the incubation on a rotor at the speed of 50 rmp. (B.) Demonstrate a duplication of the same experiment with the incubation in the incubator (no aeration given). Red line: growth percentages for the two drug combined culture calculated against LB control, as compared to the expected growth rate for an additive effect (shown in purple). Black line with squares is used for VAN only, black line with triangles is used for TMP only, black line with diamonds is control of LB only. The x-axis is used to plot the variable of cell density.

The killing effect of the synergy in wild-type *E. coli*. A time kill kinetic assay of VAN and TMP was used to demonstrate killing, with the reduction of viable cell counts of greater than 3 logs for duration of 8 hours. (Figure 3-4) VAN at 100 μg/ml on a wild-type strain essentially has no effect compared to the control grown up in LB alone. TMP at 0.15 μg/ml exhibited bacteriostatic effect for the first 4 hours, but was not sufficient to limit the growth later on. The combination showed a distinctively different kinetic where a significant reduction in viable cell counts was observed.

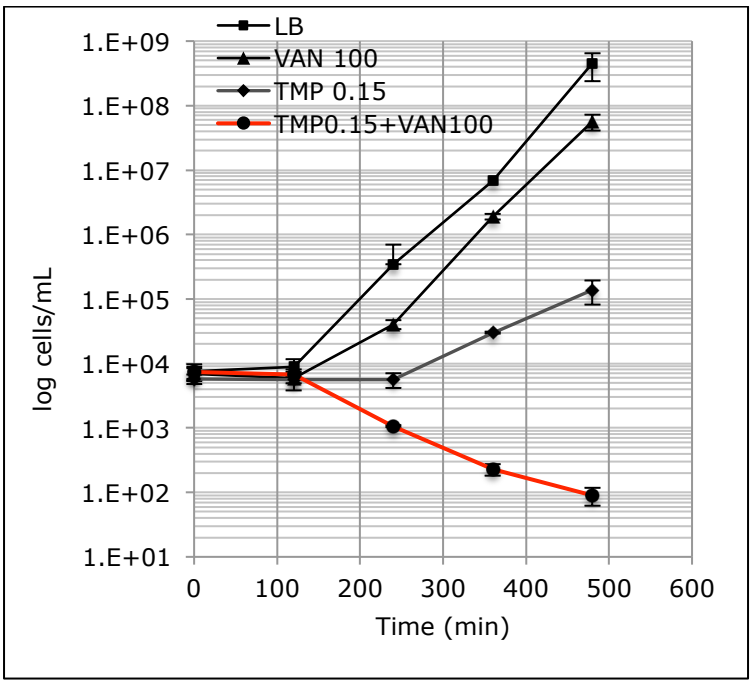

FIG 3-4. Time kill kinetics of vancomycin (100 µg/ml), trimethoprim (0.15 µg/ml), and the two drugs combined in wild-type *E. coli*, compared to a no drug control of LB only. Cultures were started with an inoculation of $1x10^4$ cells/ml and viable cells were measured by plating every two hours for eight hours. Viable cell counts were plotted against time durations in a semi-logarithmic graph.

DISCUSSION

The proliferation of multi-drug resistant bacteria is a major problem in public health. The challenge is to find new approaches to overcome antibiotic resistance, and particularly antibiotic resistant Gram-negative bacteria, whose outer membrane already precludes the use of many drugs that are effective against Gram-positive pathogens. Thus, some investigators are focusing on natural products derived from nonconventional sources, such as plants (8, 9), marine microorganisms (10), and insects (11). Another strategy involves making new chemical derivatives of existing antibiotics, based on rational design of the compounds using information such as

the 3D structure of the antibiotic (12, 13), or linking drugs to peptides that can pass through membranes to facilitate their entry into the cell (14).

Combinatorial strategies for antibiotic use offer an expanded repertoire of drug therapies (15). Of particular interest are potentiators of existing antibiotics, or "codrugs", a number of which have been used in both laboratory (16-21), and clinical (22-24) settings. Also, Collins and coworkers have demonstrated that specific metabolites can potentiate aminoglycosides acting on *E. coli* and *Staphylociccus aureus* biofilms (25). One desired potentiator would sensitize Gram-negative bacteria to vancomycin (VAN), a glycopeptide antibiotic that blocks peptidoglycan polymerization by binding to the peptidyl-D-alanyl-alanine termini of peptidoglycan precursors (26), but that cannot penetrate the outer membrane (1,27). In this regard, Collins and coworkers have shown that silver sensitizes *E. coli* to a series of antibiotics, including VAN (28), and Wink and coworkers have reported that EDTA potentiates the combination of VAN plus thymol (29). In the work reported here we utilized mutant *E. coli* strain (*dcd*) that have increased sensitivity to VAN to study pairwise synergies between VAN and a set of antibiotics (see Table 3s-1). Despite the uncertainty of exact mechanism of the synergy, there are a few suggestions can be made as contributing factors to the killing. One possibility is that a very small percentage of exogenous VAN does enter the cell, and this can act synergistically with certain other processes, such of the deprivation of thymidine and its result in "thymineless death" (30), which the situation can be exacerbated in DCD-deficient cells by the addition of cytidine (31). Collins and coworkers have postulated that bactericidal antibiotics ultimately generate hydroxyl radicals that damage DNA, resulting in cell killing (7). Thus, if small amounts of

VAN generate some DNA damage, that may be synergistic with the DNA damage caused by the partial thymineless state of DCD-deficient cells. This predicts that agents that directly damage DNA will show strong synergy with VAN, as well as agents that starve the cell of thymidine, such as trimethoprim (TMP). In deed, the synergy between VAN and TMP is strong enough for killing even in wild-type *E. coli*. The implication of the two approved drugs that are already in use does offer some advantages over screening for new treatment remedy, as the former can be applied in a clinical setting with far less delays.

CHAPTER 3 Bibliography

1. Nikaido, H. 1989. Outermembrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. 33:1831-1836.
2. Lockhardt, S. R., Abramson, M. A., Beekmann, S. E., Gallagher, G., Riedel, S., Diekema, D. J., Quinn, J. P., and Doern, G. V. 2007. Antimicrobial resistance among gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004. J. Clin. Microbiol. 45:3352-3359.
3. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., and Mori, H. 2006. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: The Keio collection. Mol. Sys. Biol. 2:2006.0008.
4. Datsenko, K. A., and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 97:6640-6645.
5. Miller, J. H. 1992. A short course in bacterial genetics: A laboratory manual and handbook for *Escherichia coli* and related bacteria, p. 194-195. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
6. Yeh, P., Tschumi, A. I., and Kishony, R. 2006. Functional classification of drugs by properties of their pairwise interactions. Nature Genetics. 28:489-494.

7. Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130:797-810.
8. Appendino, G., Gibbons, S., Giana, A., Pagani, A., Grassi, G., Stavri, M., Smith E., and Rahman, M. M. 2008. Antibacterial cannabinoids from *Cannabis sativa*: a structure-activity study. J. Nat. Prod. 71:1427-1430.
9. Shiu, W. K., Rahman, M. M., Curry, J., Stapleton, P., Zioh, M., Malkinson, J. P., and Gibbons, S. 2012. Antibacterial acylphloroglucinols from *Hypericum olympicum*. J. Nat. Prod. 75:336-343.
10. Jang, K. H., Nam, S. J., Locke, J. B., Kauffman, CA, Beatty, D. S., Paul, LA., and Fenical, W. 2013. Anthracimycin, a potent anthrax antibiotic from a marine-derived actinomycete. Angew. Chem. Int. Ed. 52:7822-7824.
11. Freitak, D., Knorr, E., Vogel, H., and Vilcinskas, A. 2012. Gender-and stressor-specific microRNA expression in *Tribolium castaneum*. Biology Letters 8:308-311.
12. Lee, R. E., Hurdle, J. G., Liu, J., Bruhn, D. F., Sherman, M. S., Vaddady, P. K., Zheng, Z., Qi, J., Akbergenov, R., Das, S., Madhura, D. B., Rathi, C., Trivedi, A., Villellas, C., Lee, R. B., Rakesh, Waidyarachchi, S. L., Sun, D., McNeil, M. R., Ainsa, J. A., Boshoff, H. I., Gonzalez-Juarrero, M., Meibohm, B., Boettger, E. C., and Lenaerta, A. J. 2014. Spectinamidea: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. Nat. Med. 20:152-158.
13. Carney, D. W., Schmitz, K. R., Truong, J. V., Sauer, R. T., and Sello, J. 2014. Restriction of the conformational dynamics of the cyclic

acyldepsipeptide antibiotics improves their antibacterial activity. J. Am. Chem. Soc. 136:1922-1929.

14. Geller, B. L., and Greenberg, D. E. 2014. Peptide-conjugated phosphorodiamidate morpholino oligomers: a new strategy for tackling antibiotic resistance. Ther. Deliv. 5:243-245.
15. Cottarel, G., and Wierzbowski, J. 2007. Combination drugs, an emerging option for antibacterial therapy. Trends in Biotech. 25:547-555.
16. Lomovskaya, O., Warren, M. S., Lee, A., Galazzo, J., Fronko, R., Lee, M., Blais, J., Cho, D., Chamberland, S., Renau, T., Ishida, H., and Lee, V. J. 2001. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination theraby. Antimicrob. Agents Chemother. 45:105-116.
17. Nelson, M. L., and Levy, S. B. 1999. Reversal of tetracycline resistance by different bacterial tetracycline resistance determinants by an inhibitor of the Tet(B) antiport protein. Antimicrob. Agents Chemother. 43:1719-1724.
18. Conlon, B. P., Nakayasu, E. S., Flack, L. E., LaFleur M. D., Isabella, V. M., Coleman, K., Leonard, S. N., Smith, R. D., Adkins, J. N., and Lewis, K. 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature 503:365-370.
19. Hu, Y., and Coates, A. R. 2013. Enhancement by novel anti-methicillin-resistant *Staphylococcus aureus* compound HT61 of the activity of neomycin, gentamicin, mupirocin, and chlorhedidine: in vitro and in vivo studies. J. Antimicrobial Chemother. 68:374-384.

20. Ejim, L., Farha, M. A., Falconer, S. B., Wildenhain, J., Coombes, B. K., Tyers, M., Brown, E. D., and Wright, G. 2011. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. Nat. Chem. Biol. 7:348-350.
21. Marks, L. R., Clementi, E. A., and Hakansson, A. 2012. The human milk protein-lipid complex HAMLET sensitizes bacterial pathogens to traditional antimicrobial agents. PLOS ONE 7:e43514.
22. Buynak, J. D. 2006. Understanding the longevity of the β-lactam antibiotics and of antibiotic/β-lactamase inhibitor combinations. Biochemical Pharmacology 71:930-940.
23. Hidaka, M., Kawashima, K., Yoshida, M., and Mitsuhashi, S. 1992. Inactivation of new carbapenem antibiotics by dehydropeptidase-1 from porcine and human renal cortex. J. Antimicrob. Chemother. 30:129-134.
24. Hoffman, J., Trimble, J., and Brophy, G. M. 2009. Safety of imipenem/cilastin in neurocritical care patients. Neurocrit. Care 10:403-407.
25. Allison, K. R., Brynildsen, M. P., and Collins, J. J. 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473:216-220.
26. Barna, J. C., and Williams, D. H. 1984. The structure and mode of action of glycopeptides antibiotics of the vancomycin group. Annu. Rev. Microbiol. 38:339-357.
27. Pages, J. M., James, C. E., and Winterhalter, M. 2008. The porin and the permeating antibiotic: A selective diffusion barrier in Gram-negative bacteria. Nat. Rev. Microbiol. 6:893-903.

28. Morones-Ramirez, J. R., Winkler, J. A., Spina, C. S., and Collins, J. J. 2013. Silver enhances antibiotic activity against Gram negative bacteria. Science Transl. Med. 5:190ra81.

29. Hamoud, R., Zimmermann, S., Reichling, J., and Wink, M. 2013. Synergistic interactions in two-drug and three drug combinations (thymol, EDTA, and vancomycin) against multi drug resistant bacteria including *E. coli*. doi: 10.1016/j. phymed.

30. Ohkawa, T. 1975. Studies of intracellular thymidine nucleotides. Thymineless death and the recovery after re-addition of thymine in *Escherichia coli* K12. Eur. J. Biochem. 60:57-66.

31. Kang, T. M., Yuan, J., Zhou, A., Beppler, C., and Miller, J. H. 2014. "Deoxycytidine Deaminase-deficient *Escherichia coli* Strains Display Acute Sensitivity to Cytidine, Adenosine, and Guanosine, and Increased Sensitivity to a Range of Antibiotics, Including Vancomycin." J. Bacteriol. 196(11):1095.