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Genetic analysis of zwittermicin A resistance in *Escherichia coli*: effects on membrane potential and RNA polymerase

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Summary

Zwittermicin A is a novel aminopolyol antibiotic that represents a new structural class of antibiotic and has diverse biological activities, including the suppression of plant disease and the ability to inhibit prokaryotic and eukaryotic cells. To enhance our fundamental understanding and applications of zwittermicin A, we elucidated mechanisms of zwittermicin A resistance in *Escherichia coli*. Two classes of zwittermicin A-resistant mutants of *E. coli* were selected and characterized. One class included mutants altered in *hemA*, *hemB*, *hemL*, *ubi*, *cydAB* or *atp*, which were defective in generating a proton motive force (PMF) and resistant to aminoglycosides. The mutant analysis, coupled with physiological data, indicated an association between the electrical membrane potential ($\Delta\Psi$) component of PMF and zwittermicin A sensitivity. A second class of zwittermicin A-resistant mutants was aminoglycoside sensitive and was affected in *rpoB* and *rpoC*, genes that encode subunits of RNA polymerase. The *rpoB* and *rpoC* mutants suggested that zwittermicin A might inhibit transcription, DNA replication, DNA gyrase or topoisomerase I; however, we found no further evidence to support any of these as the target for zwittermicin A. This study elucidated the genetic mechanisms of zwittermicin A resistance in *E. coli*. The results suggest that $\Delta\Psi$ drives zwittermicin A uptake, and that, unlike other antibiotics for which resistance maps in *rpoB* or *rpoC*, zwittermicin A does not cause the rapid cessation of DNA or RNA synthesis, suggesting a unique mechanism of antibiosis.

Introduction

Zwittermicin A is a novel, broad-spectrum antibiotic produced by *Bacillus cereus* (He *et al*., 1994; Silo-Suh, 1994; Stabb *et al*., 1994; Milner *et al*., 1995). Zwittermicin A inhibits the growth of eukaryotic and prokaryotic microorganisms and is particularly active against enteric bacteria, such as *Escherichia coli*, and plant pathogenic oomycetes belonging to the genera *Phytophthora*, *Pythium* and *Aphanomyces* (Silo-Suh, 1994). Zwittermicin A also contributes to the ability of *B. cereus* to suppress certain plant diseases (Silo-Suh *et al*., 1994). Zwittermicin A producers are ubiquitous in soil (Stabb *et al*., 1994), suggesting that it may be an ecologically important antibiotic. The unique structure of zwittermicin A (He *et al*., 1994, Fig. 1) may reflect a novel mechanism of inhibition in target organisms, which could make zwittermicin A a valuable new tool in cell biology. Based on its broad target range and inhibition of plant pathogens, zwittermicin A may also be useful in agriculture or medicine. The utility of zwittermicin A will be enhanced by an understanding of resistance mechanisms in target organisms.

Current knowledge of zwittermicin A, including its structure and activity spectrum, does not suggest the mechanism of resistance. The diversity of microorganisms inhibited by zwittermicin A may indicate an evolutionarily conserved target and uptake systems, but does not implicate any particular one. As a small, linear aminopolyol, zwittermicin A constitutes a new class of antibiotic and, therefore, resistance mechanisms cannot be predicted from the structure. Zwittermicin A shares certain structural features with other antibiotics, but these reflect diverse structures and modes of action, including amine-containing ionophores, minor groove-binding basic oligopeptides, the polycationic transcription inhibitor, chitosan, the arginine analogue, canavanine, and aminoglycoside antibiotics, which inhibit translation and interfere with membrane integrity (Hadwinger *et al*., 1986; Leuba and Stossel, 1986; Van Bambeke *et al*., 1993; Lancini *et al*., 1995). Thus, it is difficult to predict, based on the spectrum of activity and structure, the cellular target for zwittermicin A, the mechanism by which it reaches its target or the basis for zwittermicin A resistance.

The goal of this study was to determine genetic mechanisms of resistance to zwittermicin A. We present the characterization of two classes of zwittermicin A-resistant mutants of *E. coli*. One class is affected in genes whose products are involved in maintaining a membrane potential, and the other is affected in genes encoding subunits of RNA polymerase.

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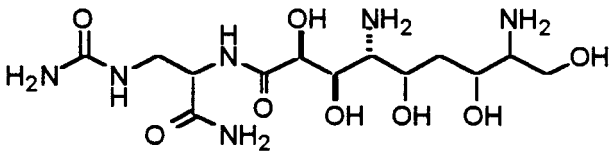

Fig. 1. Chemical structure of zwittermicin A.

Results

The goal of this study was to investigate mechanisms of zwittermicin A resistance in prokaryotes. Our approach was to select and characterize zwittermicin A-resistant mutants of *E. coli*. Mutants of strain MG1655 resistant to zwittermicin A arose spontaneously at a frequency of 2×10^{-8} and formed small colonies. Twelve independent mutations were mapped by determining the co-transduction frequency of zwittermicin A resistance loci and Tn*10* markers of known location on the *E. coli* chromosome (Singer *et al*., 1989). The mutations conferring zwittermicin A resistance mapped to six loci, at 4, 9, 17, 27, 83 and 87 map units on the *E. coli* chromosome (Table 1).

Mutants affected in haem biosynthesis

Zwittermicin A resistance in mutant MGZ10 mapped to the region at 4 map units on the *E. coli* chromosome (Table 1). We isolated plasmids that restored a large colony phenotype and zwittermicin A sensitivity to strain MGZ10 Mu *c*ts, and Southern blot analysis indicated that these plasmids contained inserts from the 4 map unit region (data not shown). The region overlapped by three of these clones included *hemL* (Fig. 2A), which encodes glutamate 1-semialdehyde aminotransferase, an enzyme in the haem biosynthetic pathway (Ilag *et al*., 1991). Similarly, the mutations in MGZ01 and MGZ09 mapped near *hemA* at 27 map units, and the mutation in MGZ11 mapped near *hemB* at 9 map units (Table 1). *hemA* and *hemB* also encode enzymes in the haem biosynthetic pathway, glutamyl-tRNA reductase and δ-aminolaevulinic acid (ALA) dehydratase respectively. Plasmids carrying *hemA* (Chen *et al*., 1994) or *hemB* restored zwittermicin A sensitivity and wild-type colony morphology on the putative *hemA* or *hemB* mutants respectively; control plasmids that contained incomplete or interrupted *hemA* or *hemB* or no insert did not restore these phenotypes (Fig. 2B and C). The enzymes encoded by *hemL* and *hemA* contribute to the synthesis of the haem precursor ALA, so, to test whether ALA deficiency caused zwittermicin A resistance in MGZ10, MGZ01 and MGZ09, we added ALA to the growth medium, and this restored zwittermicin A sensitivity and wild-type colony morphology to these mutants. These data indicate that zwittermicin A resistance is conferred by mutations in *hemL*, *hemA* or *hemB* that result in a deficiency in haem biosynthesis.

Mutants affected in the cytochrome d *terminal oxidase*

The mutation conferring zwittermicin A resistance on strain

Table 1. Mapping of zwittermicin A resistance loci by P1 transduction.

Zm^r strain	Tn*10* source[a]	Chromosomal derivation from MG1655[a]	Tn*10* location (map units)[a]	Per cent co-transduction[b] of Zm^s and Tc^r	Per cent co-transduction[b] of Zm^r and Tc^r
Spontaneous Zm^r mutants (aminoglycoside resistant)					
MGZ10	CAG12025	*zad-220*::Tn*10*	3.2	51	40
MGZ10	CAG18436	*zae-502*::Tn*10*	4.4	23	15
MGZ01	CAG12016	*zcg-3060*::Tn*10*	27.4	76	79
MGZ09	CAG12016	*zcg-3060*:Tn*10*	27.4	66	63
MGZ11	CAG18091	*proC zaj-3053*::Tn*10*	9.0	97	97
MGZ06	CAG12147	*nad57*::Tn*10*	16.9	96	84
MGZ05	CAG18496	*fadAB101*::Tn*10*	86.7	97	63
MGZ07	CAG18496	*fadAB101*::Tn*10*	86.7	97	81
MGZ12	CAG18496	*fadAB101*::Tn*10*	86.7	99	96
MGZ02	CAG18501	*zie-296*:Tn*10*	83.5	82	49
MGZ03	CAG18501	*zie-296*:Tn*10*	83.5	88	40
MGZ04	CAG18501	*zie-296*:Tn*10*	83.5	83	47
MGZ08	CAG18501	*zie-296*:Tn*10*	83.5	85	44
Zm^r mutants from EMS-treated culture (aminoglycoside sensitive)					
MGZ201	CAG18500	*thi-39*::Tn*10*	90.3	95	91
MGZ201	CAG12185	*argE86*::Tn*10*	89.4	48	21
MGZ202	CAG18500	*thi-39*::Tn*10*	90.3	98	ND[c]
MGZ203	CAG18500	*thi-39*::Tn*10*	90.3	98	97

a. Originally from Singer *et al.* (1989), updated by Berlyn *et al.* (1996).
b. Co-transduction was measured with P1 *vir*. Co-transduction of zwittermicin-sensitive (Zm^s) phenotype and tetracycline-resistant (Tc^r) phenotype was measured using a Zm^{s}Tc^r donor and a zwittermicin-resistant (Zm^r) mutant recipient. Co-transduction of Zm^r and Tc^r was measured using a Zm^{r}Tc^r donor and the wild-type MG1655 as recipient. In each experiment, tetracycline was added to plates to select for transduction of the Tn*10* marker, and co-transduction of Zm^r or Zm^s phenotypes was screened.
c. ND, not determined; P1 *vir* did not lyse the appropriate donor strain.

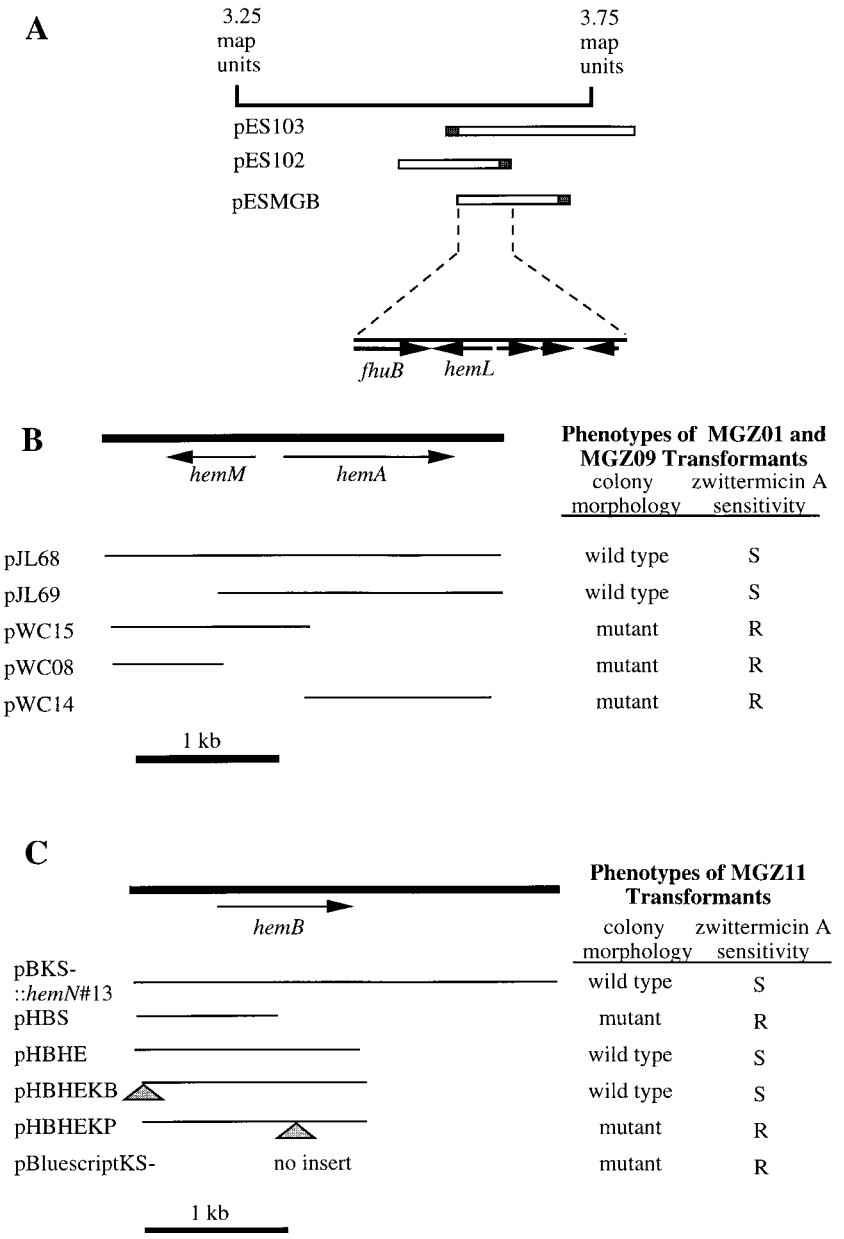

Fig. 2. A. Placement of pES102, pES103 and pESMGB inserts relative to the *E. coli* chromosome. The solid black line (top) represents the region of the *E. coli* chromosome from 3.25–3.75 map units. The portions of the chromosome contained on the inserts of pES103, pES102 and pESMGB are shown as boxes aligned under the corresponding region of the chromosome. The shaded portions of the boxes represent regions aligned by sequence identity to the corresponding chromosomal region; the remaining, unshaded portions are inferred based on insert size. The region overlapped by all three plasmids is expanded, and open reading frames identified in the sequence of this region (Fujita *et al.*, 1994) are indicated as arrows and labelled if genes have been assigned.
B and C. Characteristics of zwittermicin A-resistant mutants transformed with plasmids containing *hemA* or *hemB*, and flanking regions. The thick line represents the chromosomal region. *hemM*, *hemA* and *hemB* open reading frames are represented as arrows. The extent of the insert in each plasmid is shown with a thin line beneath the corresponding chromosomal region (B taken from Chen *et al.*, 1994). Shaded triangles indicate sites at which the Kan^r cassette from pUC4K has been inserted. Colony morphology of transformants was either mutant (small) or wild type (large). Zwittermicin A sensitivity was tested by the radial streak method. S indicates zwittermicin A sensitive, and R indicates zwittermicin A resistant.

MGZ06 was localized to the region at 17 map units on the *E. coli* chromosome (Table 1), close to *cydAB*, an operon encoding subunits of the *E. coli* cytochrome *d* terminal oxidase complex (Green and Gennis, 1983; Green *et al.*, 1984). To test whether a deficiency in *cyd* production results in zwittermicin A resistance, we assayed *cyd*$^-$ mutant GR84N (Green *et al.*, 1984) and found that it was resistant to zwittermicin A. Furthermore, zwittermicin A sensitivity and wild-type colony morphology were restored to MGZ06 by plasmid pNG2, a derivative of pBR322 that carries the *cydAB* operon (Green *et al.*, 1984), whereas pBR322 did not affect these phenotypes. These data indicate that the elimination of *cyd* confers zwittermicin A resistance and that the zwittermicin A resistance of MGZ06 is caused by a lesion in *cyd*.

Mutants affected in ubiquinone biosynthesis

The mutations conferring zwittermicin A resistance on mutants MGZ05, MGZ07 and MGZ12 were mapped to the region at 87 map units on the *E. coli* chromosome (Table 1). Based on co-transduction frequencies with other nearby markers (data not shown), the mutation in MGZ05 was localized counterclockwise from *fadAB*, in the vicinity of *ubiB* and *ubiD* (Cox *et al.*, 1969; Daniels *et al.*, 1992), which encode enzymes involved in the biosynthesis of

the electron carrier, ubiquinone (Young *et al*., 1973). Consistent with a disruption of ubiquinone biosynthesis, mutants MGZ05, MGZ07 and MGZ12 did not grow under ubiquinone-dependent conditions, aerobically with malate or succinate as a carbon source, but grew well under conditions that do not require ubiquinone, aerobically or anaerobically on glucose or anaerobically on glycerol and nitrate (Cox *et al*., 1968; Lin and Kuritzkes, 1987; Wissenbach *et al*., 1990). Furthermore, we found that a previously described *ubiD* mutant, AN66 (Cox *et al*., 1969), was resistant to zwittermicin A, while its parent strain, AN2154, was sensitive to zwittermicin A, demonstrating that deficiency in ubiquinone production confers zwittermicin A resistance.

Mutants in membrane-bound energy-generating ATPase

The mutations conferring zwittermicin A resistance on mutants MGZ02, MGZ03, MGZ04 and MGZ08 mapped near the *atp* locus at 83 map units on the *E. coli* chromosome (Table 1). MGZ02, MGZ03, MGZ04 and MGZ08 grew with glucose but not with succinate or malate as the carbon source, which is a phenotype typical of *atp* mutants (Adler and Rosen, 1976), and pAP55, which carries the *atp* operon (Brusilow *et al*., 1983), restored zwittermicin A sensitivity and wild-type colony morphology to these mutants, while the vector alone did not affect these phenotypes.

Some previously described *atp* mutants are 'leaky', deregulating the flow of protons through the membrane, and are decreased in the uptake of various solutes (Rosen, 1973; Damper and Epstein, 1981; Humbert and Altendorf, 1989). One such 'leaky' mutant, TK1207 (Damper and Epstein, 1981), was resistant to zwittermicin A, and its parent strain, FRAG5, was sensitive to zwittermicin A. The proton 'leak' in mutants such as TK1207 can be blocked with DCCD (Rosen, 1973; Damper and Epstein, 1981), and DCCD dramatically increased the zwittermicin A sensitivity of strains MGZ02, MGZ03, MGZ04 and MGZ08, but had little effect on the zwittermicin A sensitivity of the wild-type strain MG1655 or the *hemB* mutant MGZ11 (Table 2). These data indicate that strains MGZ02, MGZ03, MGZ04 and MGZ08 are *atp* mutants and are zwittermicin A resistant, because they deregulate the flow of protons through the cytoplasmic membrane.

Table 2. Effect of DCCD on minimal inhibitory concentration of zwittermicin A.

Strain	MIC zwittermicin A ($\mu g\,ml^{-1}$)[a] without DCCD[b]	MIC zwittermicin A ($\mu g\,ml^{-1}$)[a] with DCCD
MG1655	40	40
MGZ02	250	40
MGZ03	275	50
MGZ04	275	50
MGZ08	250	40
MGZ11	150	125

a. Since DCCD was dissolved in ethanol, all treatments contained 1% ethanol.
b. MIC was determined, with or without DCCD (0.5 mM), by measuring the lowest concentration of zwittermicin A required to prevent visible growth after inoculating $\approx 10^5$ cells in 1 ml of MHMT8.1 and shaking in a 28°C incubator for 24 h.

Association between zwittermicin A resistance and membrane potential

Mutants, such as those described above, with 'leaky' membrane-bound ATPases or alterations in electron transport functions, such as haem, cytochrome or ubiquinone biosynthesis, are reduced in proton motive force (PMF) and its electrical membrane potential ($\Delta\Psi$) component. We tested the zwittermicin A sensitivity of wild-type *E. coli* strain MG1655 under conditions that decrease $\Delta\Psi$, including increased osmolarity, lowered external pH and the addition of the uncoupler 2,4-dinitrophenol (Damper and Epstein, 1981), and found that all of these conditions increased the minimal inhibitory concentration (MIC) of zwittermicin A 1.7- to 20-fold (Fig. 3). Decreases in external pH decrease $\Delta\Psi$ but increase PMF and its ΔpH component (Harold and Maloney, 1996), so the decreases in zwittermicin A sensitivity, in both the mutants and the culture conditions, are consistently associated with decreases in $\Delta\Psi$, not in PMF. Furthermore, decreased $\Delta\Psi$ is correlated with decreased uptake of and low-level resistance to aminoglycoside antibiotics (Damper and Epstein, 1981), and all 12 Zm^r mutants were resistant to low levels of the aminoglycosides, streptomycin, gentamicin and kanamycin, while remaining sensitive to non-aminoglycosides, such as tetracycline, chloramphenicol and ampicillin. To test the relationship between zwittermicin A sensitivity and $\Delta\Psi$ quantitatively, we measured sensitivity to aminoglycosides as an indirect assessment of $\Delta\Psi$. This approach was based on the finding (Damper and Epstein, 1981) that the logarithm of the minimal inhibitory concentrations [log(MIC)] of dihydrostreptomycin and of kanamycin are linearly and inversely related to the magnitude of $\Delta\Psi$. We found that the log(MIC) of zwittermicin A was directly correlated with the log(MIC) of dihydrostreptomycin ($r^2 = 0.91$) (Fig. 3) and with the log(MIC) of kanamycin among 12 culture conditions or mutants ($r^2 = 0.84$) (data not shown). Taken together, the data indicate an inverse relationship between $\Delta\Psi$ and zwittermicin A resistance.

Isolation of zwittermicin A-resistant, aminoglycoside-sensitive mutants

Mutations that lower $\Delta\Psi$ decrease aminoglycoside uptake and confer low-level, non-specific aminoglycoside resistance, whereas selection of high-level resistance to specific

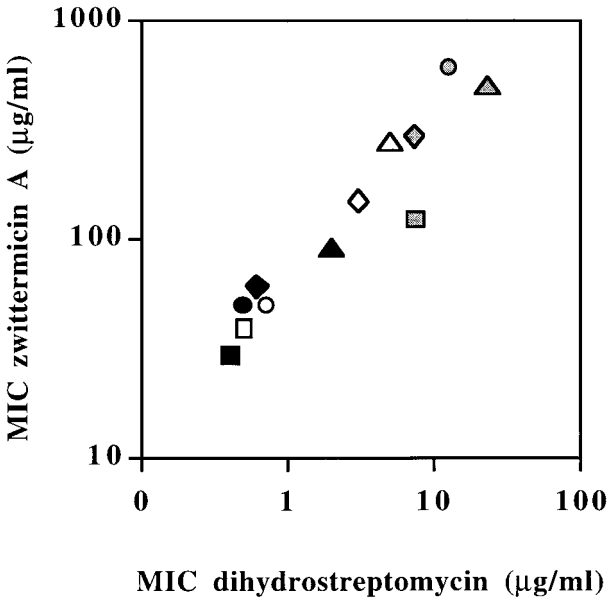

Fig. 3. Log(MIC) of dihydrostreptomycin vs. log(MIC) of zwittermicin A. Strains and culture conditions for which MICs were measured include strain MG1655 in MHMT8.1 (black square), with 300 mM sucrose (black circle), with 1 mM 2,4-dinitrophenol (black diamond), with 500 mM NaCl (shaded square), in MHMT7.1 (black triangle) and in MHMT6.1 (shaded circle); strain MGZ02 in MHMT8.1 (open triangle), with 0.5 mM DCCD (open circle) or with 500 mM NaCl (shaded triangle); strain MGZ10 in MHMT8.1 (shaded diamond) with 50 $\mu g\,ml^{-1}$ ALA (open square); and strain MGZ06 in MHMT8.1 (black triangle). MIC was determined by measuring the lowest concentration of zwittermicin A or dihydrostreptomycin required to prevent visible growth after inoculating $\approx 10^5$ cells in 1 ml of medium and shaking in a 28°C incubator for 24 h. The best linear fit to the relationship between the axes was log[(MIC) zwittermicin A] = 0.68 log[(MIC) dihydrostreptomycin] + 1.8 ($r^2 = 0.91$).

aminoglycosides results in alterations in their ribosomal target (Thorbjarnardóttir *et al*., 1978; Davis, 1987). Similarly, mutations that affected $\Delta\Psi$ also affected zwittermicin A sensitivity, possibly by affecting uptake; thus, to obtain mutants altered in the intracellular target for zwittermicin A, we selected high-level zwittermicin A resistance and screened for mutants that were aminoglycoside sensitive. Spontaneous mutants resistant to 300 $\mu g\,ml^{-1}$ zwittermicin A were not observed among 10^{10} cells plated. However, after ethylmethane sulphonate (EMS) mutagenesis, mutants resistant to this level of zwittermicin A were obtained. Fifty-six zwittermicin A-resistant mutants derived from an EMS-mutagenized culture were screened for sensitivity to streptomycin, gentamicin and kanamycin, and four mutants, each of which produced small colonies, were aminoglycoside sensitive. The colony morphology of MGZ201, MGZ202 and MGZ203 was stable in the absence of zwittermicin A, facilitating their characterization. These three mutants were sensitive to chloramphenicol and tetracycline, indicating that they were not multiple antibiotic-resistant mutants of the sort mapped to the *mar* locus (George and Levy, 1983a,b). However, MGZ201 was resistant to nalidixic acid, which inhibits DNA gyrase, and MGZ202 was resistant to rifampicin, which inhibits RNA polymerase. The mutants obtained in this selection were easily distinguishable from the mutants affected in $\Delta\Psi$, as the mutants described here were resistant to equal or higher levels of zwittermicin A but were as sensitive as the wild type to aminoglycosides (as measured by zone sizes in radial streak tests).

Mutations in rpoB *and* rpoC

The mutations conferring zwittermicin A resistance on MGZ201, MGZ202 and MGZ203 mapped to the region containing *rpoB* and *rpoC*, which encode the two largest subunits of RNA polymerase, at 90 map units on the *E. coli* chromosome (Table 1). To test whether the growth deficiency of these mutants was caused by alterations in *rpoB* or *rpoC*, we transformed the mutants with plasmids bearing wild-type copies of these genes. MGZ201 and MGZ203 formed larger colonies when transformed with pRW308, which carries wild-type *rpoC* (Weilbaecher *et al*., 1994), than when transformed with the control vector, pTrc99c. Neither pRW308 nor pRL385, which carries *rpoB* (Landick *et al*., 1990), restored wild-type colony morphology to MGZ202. Induction of expression of a plasmid-borne *rpoC* under control of the *trp/lac* promoter increased the zwittermicin A sensitivity of MGZ201 and MGZ203 (Table 3), demonstrating a correlation between *rpoC* and zwittermicin A resistance/sensitivity in these mutants. Marker exchange analysis of MGZ201, MGZ202 and MGZ203, using plasmids bearing portions of *rpoB* or *rpoC*, identified particular regions of these genes necessary for small colony morphology and zwittermicin A resistance in the mutants (Fig. 4). Subsequent sequencing of these regions revealed, in each case, a GC to AT transition typical of EMS mutagenesis (Miller, 1992), resulting in the alteration of an evolutionarily conserved amino acid residue in RNA polymerase; RpoB E1329K in MGZ202, and RpoC G916D and S1324L in MGZ201 and MGZ203 respectively (Fig. 5). Induction of expression of the *rpoC* S1324L allele, carried on plasmid p3502 (Weilbaecher *et al*., 1994), conferred increased zwittermicin A resistance on the wild type (Table 3). These data indicate that mutations affecting RNA polymerase confer resistance to zwittermicin A.

Zwittermicin A does not inhibit DNA or RNA synthesis

Mutations in the genes encoding subunits of RNA polymerase have been shown to confer resistance to antibiotics that inhibit transcription, such as rifampicin (Jin and Gross, 1988), and those that inhibit DNA gyrase and therefore DNA replication, such as novobiocin and nalidixic acid (Blanc-Potard *et al*., 1995). We tested the possibility that zwittermicin A inhibits transcription or DNA replication.

Table 3. Effect of plasmid-borne *rpoC* alleles on zwittermicin A sensitivity.

Strain	Plasmid[a]	IPTG (mM)	MIC zwittermicin A ($\mu g\,ml^{-1}$)[b]
MGZ201	pTrc99c	0	225
MGZ201	pTrc99c	25	225
MGZ201	pRW308	0	100
MGZ201	pRW308	1	80
MGZ201	pRW308	25	70
MGZ203	pTrc99c	0	250
MGZ203	pTrc99c	25	250
MGZ203	pRW308	0	175
MGZ203	pRW308	1	150
MGZ203	pRW308	25	125
MG1655	pTrc99c	0	40
MG1655	pTrc99c	25	50
MG1655	pRW308	0	40
MG1655	pRW308	25	50
MG1655	p3502	0	60
MG1655	p3502	1	80
MG1655	p3502	25	100

a. Plasmids pRW308 and p3502 contain the wild-type *rpoC* and mutant *rpoC3502* (S1324L) expressed and regulated by the *trp*/*lac* promoter and *lacI*^{q}-encoded repressor, respectively, in vector pTrc99c (Amann *et al.*, 1988).
b. MIC was determined by measuring the minimum concentration of zwittermicin A required to prevent visible growth after inoculating 10^5 cells of *E. coli* strain MG1655 in 1 ml of medium and shaking in a 28°C incubator for 24 h.

Zwittermicin A did not inhibit transcription *in vitro* (data not shown), nor did it inhibit incorporation of [^{3}H]-uridine or [^{3}H]-thymidine by growing cells (Fig. 6). In similar experiments, the growth rate (as measured by the OD_{600}) of zwittermicin A-treated cultures slowed within 15 min of zwittermicin A addition, showing that the antibiotic was active in this time frame. These results indicate that zwittermicin A does not inhibit total RNA or DNA synthesis.

Nalidixic acid causes DNA gyrase to form double-stranded breaks in DNA, inducing SOS-response genes, such as *dinD*. Unlike nalidixic acid, zwittermicin A did not induce a *dinD*::*lacZ* fusion in *E. coli*. Novobiocin causes the progressive unwinding of negatively supercoiled DNA and thereby induces certain superhelicity-sensitive promoters, such as that in the *his* operon (Blanc-Potard *et al.*, 1995). In contrast to novobiocin, zwittermicin A did not induce transcription of the *hisD*::*lacZ* fusion in *Salmonella typhimurium*.

The effects of zwittermicin A and novobiocin on bacterial growth were antagonistic (Stabb, 1997). Mutations in *topA*, which affect topoisomerase I activity, are also antagonistic to novobiocin (Hammond *et al.*, 1991), so we tested whether zwittermicin A acts on topoisomerase I. Zwittermicin A did not inhibit the unwinding of plasmid DNA by topoisomerase I *in vitro* (data not shown). Thus, despite the fact that mutations in RNA polymerase confer zwittermicin A resistance, we found no evidence that zwittermicin A affects cellular functions previously associated with RNA polymerase, including RNA synthesis, DNA synthesis or DNA gyrase or topoisomerase I activity.

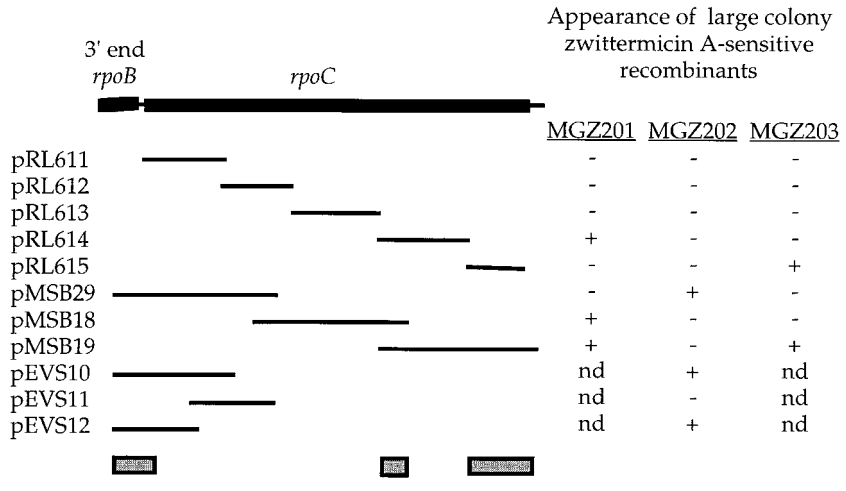

Fig. 4. Marker exchange mapping. Large colony, zwittermicin A-sensitive recombinants of strains MGZ201, MGZ202 and MGZ203 appeared when these Zm^r mutants were transformed with plasmids carrying certain portions of *rpoB* and *rpoC*. The top line represents the *E. coli* chromosome, and thick portions represent the coding regions of (from left) the 3′ end of *rpoB* and *rpoC* respectively. Plasmids are listed in the leftmost column, and the insert in each plasmid is shown as a thin line to the right of the plasmid name beneath the corresponding region of the *E. coli* chromosome. The three columns at the right indicate the stability of mutant transformants; +, indicates instability marked by the appearance of large-colony, zwittermicin A-sensitive recombinants; −, indicates the transformant stably maintained mutant colony morphology and zwittermicin A resistance. nd, not determined. The shaded boxes at the bottom indicate the regions amplified and sequenced in mutants MGZ202, MGZ201 and MGZ203 (from left to right).

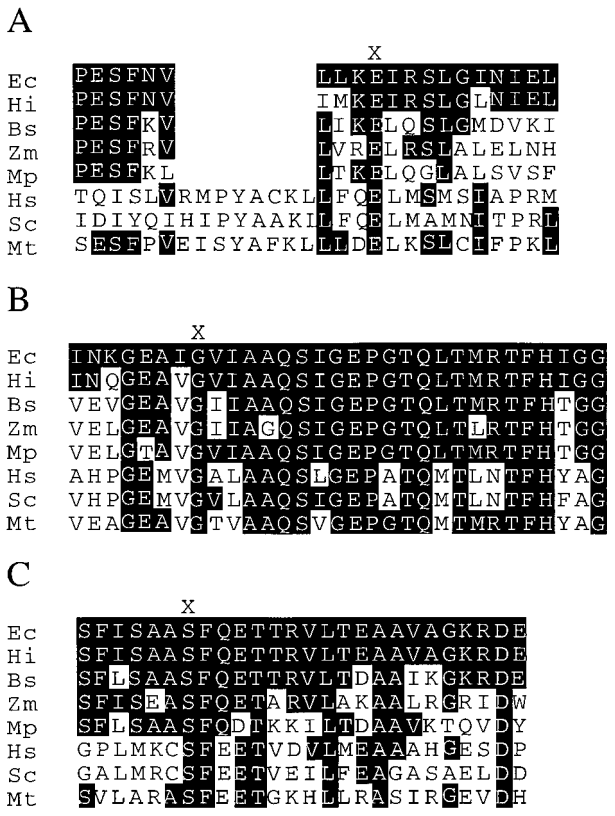

Fig. 5. Amino acids altered in zwittermicin A-resistant mutants are conserved among RNA polymerases. Amino acid sequences represent portions of RNA polymerases from the Bacteria *E. coli* (Ec), *Haemophilus influenzae* (Hi), *Bacillus subtilis* (Bs) and *Mycoplasma pneumoniae*; the chloroplasts of *Zea mays* (Zm); the nucleus (RNA polymerase II) of *Homo sapiens* (Hs) and *Saccharomyces cerevisiae* (Sc); and the Archaea *Methanobacterium thermoautotrophicum* (Mt). The amino acid sequences from *E. coli* represent RpoB residues 1320–1339 (A), RpoC residues 909–939 (B) and RpoC residues 1318–1343 (C). Shaded regions cover amino acids that are identical to the *E. coli* sequence, and gaps are shown to achieve better alignment. X marks the locations of mutant alleles; RpoB E1329 (A), RpoC G916 (B) and RpoC and S1324 (C.).

Discussion

Two distinct classes of *E. coli* mutants were resistant to zwittermicin A: one class was defective in the ability to generate PMF and was resistant to both zwittermicin A and aminoglycosides, and the second class was zwittermicin A resistant, aminoglycoside sensitive and affected in RNA polymerase. The mutants altered in PMF suggested an association between $\Delta\Psi$ and zwittermicin A sensitivity, and this was supported by other genetic and physiological lines of evidence. We propose that $\Delta\Psi$ contributes to zwittermicin A uptake. The mutants with altered RNA polymerase suggested that zwittermicin A might inhibit transcription, DNA replication, gyrase or topoisomerase activity. However, we found no evidence to indicate that any of these is the target for zwittermicin A. The mutants and the observation that zwittermicin A and novobiocin have antagonistic activities will contribute to the elucidation of the mode of action of zwittermicin A.

Association between $\Delta\Psi$ and zwittermicin A sensitivity

Zwittermicin A resistance is probably associated with decreases in $\Delta\Psi$ not decreases in PMF or ΔpH. Zwittermicin A resistance mapped to genes whose products contribute to the respiratory generation of PMF, and its $\Delta\Psi$ and ΔpH components, but it is unlikely that zwittermicin A resistance is associated with ΔpH, as $\Delta\Psi$ is the major contributor to PMF during respiratory growth at pH 8.1, the pH at which we selected for zwittermicin A resistance (Harold and Maloney, 1996). Furthermore, lowering external pH, which increases PMF and ΔpH and decreases $\Delta\Psi$ (Damper and Epstein, 1981; Harold and Maloney, 1996), increased zwittermicin A resistance (Fig. 3), indicating that zwittermicin A resistance is consistently associated with decreased $\Delta\Psi$ rather than with decreased PMF or ΔpH. Also consistent with the hypothesis that $\Delta\Psi$ is associated with zwittermicin A sensitivity is the observation

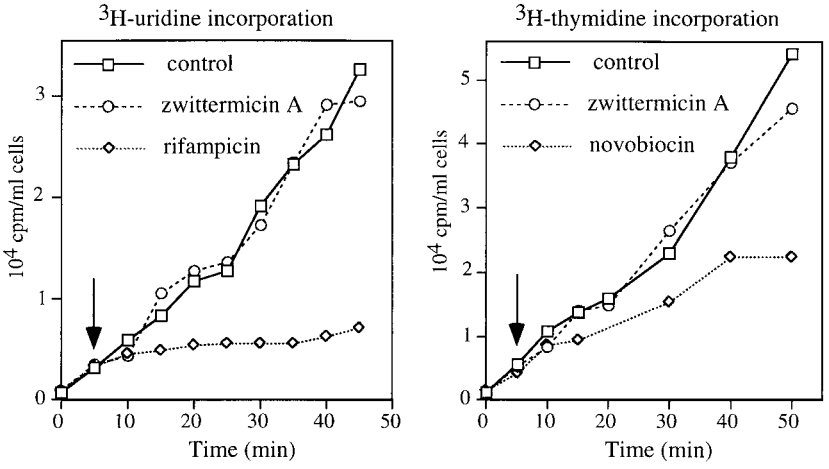

Fig. 6. Effect of antibiotics on DNA and RNA synthesis in intact *E. coli* cells. Nucleic acid synthesis was measured by the incorporation of [^{3}H]-thymidine and [^{3}H]-uridine. Novobiocin (1 mg ml^{-1}), which inhibits DNA synthesis, and rifampicin (300 μg ml^{-1}), which inhibits RNA synthesis, are included as controls. A total of 500 μg ml^{-1} zwittermicin A was added. Time of antibiotic addition is indicated by an arrow.

that, over a range of mutants and culture conditions, the level of zwittermicin A sensitivity was directly correlated with the level of aminoglycoside sensitivity (Fig. 3), and a correlation between aminoglycoside sensitivity and $\Delta\Psi$ has been demonstrated previously (Damper and Epstein, 1981). While $\Delta\Psi$ has been suggested as being involved in the sensitivity of bacteria to a number of antibiotics, direct evidence is lacking in most cases. Moreover, not all positively charged antibiotics require $\Delta\Psi$ for activity. Thus, it is significant that we have extended this mechanism of sensitivity to a new class of antibiotic.

Given the large number of genes devoted to the generation of PMF and $\Delta\Psi$, it may seem surprising that we found a relatively low frequency of spontaneous zwittermicin A resistance (2×10^{-8}); however, our selection demanded mutants deficient in $\Delta\Psi$ but still able to grow on medium not amended with a fermentable sugar. Thus, we may have selected against relatively common loss-of-function mutants in respiratory processes. Direct evidence to support this argument is that the mutant *hemL* allele in MGZ10 increases both the growth and the zwittermicin A sensitivity of this mutant when carried on a multicopy plasmid, indicating that this mutation does not confer a complete loss of function. Furthermore, the distribution of loci conferring zwittermicin A resistance and, in particular, the relatively high proportion of *ubi* and *atp* mutants is similar to the distribution of aminoglycoside-resistant mutants obtained in similar studies (Sasarman *et al*., 1968; Kanner and Gutnick, 1972; Rosen, 1973; Adler and Rosen, 1976; Bryan and Van Den Elzen, 1977; Thorbjarnardóttir *et al*., 1978; Humbert and Altendorf, 1989; Ilag *et al*., 1991; O'Neill *et al*., 1991).

The $\Delta\Psi$ normally maintained by *E. coli* consists of a relative negative charge inside the cell, which can drive the uptake of positively charged compounds electrogenically. Decreasing $\Delta\Psi$ enhances resistance to the positively charged aminoglycoside antibiotics by decreasing their electrogenically driven uptake, although the exact mechanism of uptake and the role of aminoglycoside structure and mode of action during uptake is unresolved (Damper and Epstein, 1981; Davis, 1987; Taber *et al*., 1987). It seems likely that a decrease in $\Delta\Psi$ reduces sensitivity to zwittermicin A, which is positively charged, by reducing its net uptake. However, the recent report that sensitivity to protamine, an antibiotic that inhibits cytoplasmic membrane functions, is related to $\Delta\Psi$ (Aspedon and Groisman, 1996) underscores the need to test this hypothesis directly. Whether zwittermicin A uptake is related to $\Delta\Psi$ will be tested directly when radiolabelled zwittermicin A is available. Biosynthesis or organic synthesis of labelled zwittermicin A requires either more efficient production and purification methods or more information than is currently available about the biosynthetic pathway or the stereochemistry of zwittermicin A.

RNA polymerase mutants may provide insight into the mode of action of zwittermicin A

If $\Delta\Psi$ drives zwittermicin A uptake as it does aminoglycoside uptake, then the three aminoglycoside-sensitive zwittermicin A-resistant mutants described here presumably confer resistance through alteration of the intracellular target for zwittermicin A, not by affecting uptake. Transduction and molecular characterization of these three mutants revealed an association between zwittermicin A resistance and mutations in *rpoB* or *rpoC*, genes encoding RNA polymerase subunits. Previously isolated mutations affecting RNA polymerase confer resistance to transcriptional inhibitors and/or gyrase inhibitors. In fact, our mutants, MGZ201 and MGZ202, were resistant to the transcription inhibitor rifampicin and the gyrase inhibitor nalidixic acid respectively. However, we found no evidence to show that transcription or gyrase is affected by zwittermicin A. Interestingly, novobiocin, which causes the relaxation of chromosomal DNA, reduced the activity of zwittermicin A (Stabb, 1997). Novobiocin acted additively, not antagonistically, with the aminoglycoside kanamycin (data not shown), indicating that its effects on zwittermicin A sensitivity are probably not mediated by effects on $\Delta\Psi$. Based on the lack of direct inhibition *in vitro* or *in vivo* of transcription, gyrase or topoisomerase I, zwittermicin A appears to behave differently from most antibiotics for which resistance has mapped to RNA polymerase.

The intriguing conflict between the zwittermicin A resistance of RNA polymerase mutants and our inability to detect an effect of zwittermicin A on DNA or RNA synthesis may suggest a more subtle mode of action not detected by our assays. For example, zwittermicin A might inhibit transcription in a promoter-specific fashion preventing expression of a small number of essential genes, or zwittermicin A might inhibit transcription termination in a site-specific manner. Promoter-specific transcriptional effects would not be unprecedented; the antibiotics gramicidin, netropsin and distamycin appear to inhibit or promote transcription in a context-dependent manner (Keilman *et al*., 1975; Sarkar *et al*., 1977; 1979; Bruzik *et al*., 1987). It is also possible that the mode of action of zwittermicin A is unrelated to transcription and that RNA polymerase mutants confer resistance to zwittermicin A by altering the expression of a particular gene or genes whose product(s) directly confer(s) zwittermicin A resistance. Mutations in *rpoB* have long been known to affect gene expression and confer resistance to tryptophan analogue antibiotics by altering transcription pausing and termination in the *trp* operon (Yanofsky and Horn, 1981; Fisher and Yanofsky, 1983). Four experimental approaches that will help to distinguish among these and other models are: (i) examination of gene expression in wild-type cells treated with zwittermicin A; (ii) comparison of gene expression between wild-type

and zwittermicin A-resistant *rpoB* and *rpoC* mutants; (iii) characterization of the properties (elongation and termination rates, etc.) of mutant RNA polymerases that confer zwittermicin A resistance; and (iv) sequencing a large number of resistance-conferring *rpoB* and *rpoC* alleles. The *rpoC* S1324L allele, which conferred zwittermicin A resistance, was previously isolated in a selection for mutant *rpoC* alleles that conferred altered transcriptional termination properties (Weilbaecher *et al.*, 1994). Examination of the other defined *rpoC* alleles conferring altered termination properties will determine whether zwittermicin A resistance and termination phenotypes are consistently associated with or coincidental to the S1324L allele. The outcomes of these experiments, combined with the wealth of basic understanding of *E. coli* RNA polymerase, should provide insight into the mode of action of zwittermicin A.

Experimental procedures

Bacteria, phage, plasmids and media

Lambda phages were amplified in *E. coli* LE392 (Murray *et al.*, 1977). Luria broth (LB; Miller, 1992) and 56 minimal base medium (Monod *et al.*, 1951) were prepared as described except that NaCl was added to LB at a concentration of 5.0 g l^{-1}. MHMT8.1 was prepared by amending Mueller Hinton medium (Difco Laboratories) with 40 mM MOPS and 40 mM Tris and adjusting the pH to 8.1 with NaOH. MHMT7.1 and MHMT6.1 were adjusted to pH 7.1 or 6.1, respectively, with HCl. When added to media, ALA, kanamycin, tetracycline, chloramphenicol and ampicillin were used at concentrations of 50, 40, 10, 30 and 75 μg ml^{-1} respectively. Methyl-[^{3}H]-thymidine (88.3 Ci $mmol^{-1}$) and 5,6-[^{3}H]-uridine (40.7 Ci $mmol^{-1}$) were obtained from NEN Life Science Products. Zwittermicin A was purified by cation exchange chromatography on an Amberlite IRL-50 resin (Sigma) followed by high-performance liquid chromatography (HPLC) with a cyano-bonded phase column, according to a protocol to be described elsewhere.

DNA manipulations and analysis

T4 ligase and restriction enzymes were obtained from Promega, except *Bcl*I, *Hpa*I and *Pst*I (New England Biolabs). Plasmids and lambda phage DNA were purified with Wizard Minipreps or Wizard Lambda Preps respectively (Promega). Between restriction and ligation reactions, and before transformation, DNA was recovered with the Qiaex gel extraction kit (Qiagen). The sizes of DNA inserts in pES102, pES103 and pESMGB were estimated by *Eco*RV digestion and gel electrophoresis. Southern blot analysis, electroporation-mediated transformation and DNA sequencing were performed as described previously (Milner *et al.*, 1996). For Southern blot analysis, probes were generated from λ117, λ119 and λ120 (Fujita *et al.*, 1994). Primer 5′-CCAATGTCCCGGTTTTT-3′ directed sequencing of inserts in plasmids pES102, pES103 and pESMGB. To sequence regions of *rpoB* or *rpoC*, template DNA was obtained from boiled lysates of cultures by polymerase chain reaction (PCR) amplification with *Pfu* polymerase (Stratagene), followed by gel purification of the product. In each case, the products of at least two separate amplifications were sequenced, producing identical results. The primers for amplification and sequencing of MGZ201 DNA were 5′-CATGAAGCTTGTTCTGTTGTATCTTGTGACACCG-3′ and 5′-CATGGGATCCCAG-TTTCAGTTCAGTATTACGGG-3′. The primers used to analyse MGZ202 were 5′-CGGTTTTAGTCTGCGCTTTCAG-3′, 5′-CTGATGGTTGCCGTCCACGATG-3′, 5′-TGGAGCCGGGCATGCCAGA-ATC-3′ and 5′-AGCCTGGTTACTCAGCAGCCGC-3′. The primers for the analysis of MGZ203 DNA were 5′-CGTTAACGCGGGTAGCTCCGAC-3′, 5′-CTCCCCCCATAAAAAAACCCG-CCGA-3′, 5′-TCTCCTGGAACGATGCCGCGGAGAT-3′, 5′-CGTGGTGACGTAATTTCCGACGGT-3′ and 5′-ACCAAAGCGTCTCTGGCAACCGAGT-3′. Primer synthesis and sequencing were done at the University of Wisconsin Biotechnology Center. Sequencing was conducted on an ABI Model 373A automated DNA sequencer, and comparison of DNA sequences was conducted using the BLASTN algorithm (Altschul *et al.*, 1990).

Plasmid construction

Plasmids pES102, pES103 and pESMGB were generated by the *in vivo* cloning method described by Groisman and Casadaban (1986). pES102 and pES103 contain inserts derived from MGZ10 Mu *c*ts, and the insert in pESMGB was derived from MG1655 Mu *c*ts. We determined that these transformants were increased in sensitivity to zwittermicin A by both radial streak tests and determination of MICs. The inserts in these plasmids were identified by determining the insert sizes, sequencing one end of each insert and aligning the sequences with the *E. coli* chromosome (Fujita *et al.*, 1994). Plasmids pHBS and pHBHE were deletion derivatives of plasmid pBKS–::*hem*N#13, which contains the same 3.1 kb *Bam*H1 fragment carrying *hemB* as pUC18ALABam3 (O'Neill *et al.*, 1991) inserted in pBluescriptKS–, and were generated by digesting pBKS–::*hem*N#13 with *Sal*I, and *Hpa*I and *Eco*RV, respectively, and self-ligating the resulting fragments. Plasmids pHBHEKP and pHBHEKB contain the Kan^r cassette from pUC4K (Vieira and Messing, 1982) inserted in pHBHE at the *Pst*I and *Bam*HI sites respectively. Plasmids pEVS10, pEVS11 and pEVS12 were generated by digesting pMSB29 with *Bcl*I and *Bam*HI, *Eco*RI and *Pvu*II, respectively, and self-ligating the resulting fragments to create deletion derivatives of pMSB29. Plasmids, with the exception of pES102, pES103 and pESMGB, were maintained in strain DH5α (Hanahan, 1983).

Assays of antibiotic sensitivity

Antibiotic sensitivity was tested qualitatively in radial streak tests (Milner *et al.*, 1996) on MHMT8.1 plates with antibiotics added to filter disks in the following amounts: zwittermicin A (150 μg), tetracycline (5 μg), chloramphenicol (10 μg), ampicillin (15 μg), gentamicin (2 μg), kanamycin (5 μg), streptomycin (5 μg), nalidixic acid (25 μg) or rifampicin (75 μg). Sensitivity to zwittermicin A, dihydrostreptomycin or kanamycin was tested semiquantitatively by a minimal inhibitory concentration (MIC) test. Approximately 10^5 cells were added to 1 ml of broth (MHMT8.1 unless otherwise stated) containing the appropriate antibiotic. The cultures were shaken at 28°C for 24 h, and the lowest concentration of antibiotic at

which no growth was visible was considered the MIC. The reported MICs reflect typical results of at least three independent determinations.

Selection of zwittermicin A-resistant mutants

We selected mutants in MHMT8.1 at 28°C because MG1655 appeared to be most sensitive to zwittermicin A under these conditions. A single colony of MG1655 was used to inoculate MHMT8.1 broth (in 17 separate experiments). The culture was shaken for 8–12 h in a 28°C incubator and then spread on solid medium containing 150 μg ml^{-1} zwittermicin A, and the plates were incubated for 48 h. Selection of zwittermicin A-resistant mutants after mutagenesis was performed as described above, except that 300 μg ml^{-1} zwittermicin A was used. Mutagenesis with EMS was performed as described previously (Miller, 1992), resulting in a 40% reduction in cfus after EMS exposure.

Assays of antibiotic activities

To test the ability of antibiotics to induce *E. coli* dinD1::Mu d1(Ap^r *lac*) or *S. typhimurium* hisD::Mu d1(Ap^r *lac*) fusions, strains PN104 (Nurse *et al*., 1991) and TT7692, respectively, were spread on LB plates containing 40 μg ml^{-1} Xgal. Antibiotic-containing filter disks were placed on the plates, which were incubated at 37°C for 24 h. A zone of blue, darker than the lawn, surrounding a zone of inhibition was scored as induction. Inhibition of transcription by *E. coli* RNA polymerase holoenzyme was tested *in vitro* as described previously (Weilbaecher *et al*., 1994), with or without the addition of zwittermicin A (300 μg ml^{-1}). Briefly, a linear DNA fragment carrying the bacteriophage T7A1 promoter and the Rho-independent T7 early terminator served as the template, and transcription products containing [^{32}P]-GTP were visualized on gels. Zwittermicin A was preincubated with either the DNA template or the RNA polymerase enzyme with similar results. Inhibition of topoisomerase I activity was assayed *in vitro* as described previously (Burton and Kaguni, 1997), using cell-free lysate from an *E. coli topA* overexpresser as a source of topoisomerase I and supercoiled pUC18 DNA as a substrate, with or without zwittermicin A (300 μg ml^{-1}). Topoisomerase I-containing lysate was generously provided by Neil Bowlby, Michigan State University. To measure the effect of antibiotics on the incorporation of methyl-[^{3}H]-thymidine or 5,6-[^{3}H]-uridine by whole cells, an early log phase (A_{600} 0.04–0.07) culture of MG1655 grown in MHMT8.1 at 28°C was split into parallel 11 ml cultures, label (0.25 μCi ml^{-1} uridine or 0.33 μCi ml^{-1} thymidine) was added to each culture and antibiotics were added to cultures after 5 min. At intervals, 1 ml of culture was removed and placed on ice, cells were pelleted and washed in 1 ml of MHMT8.1, the pellet was resuspended in 20 μl of MHMT8.1, spotted onto 24 mm glass GF/A filters (Whatman) and the filters were dried and counted in a Beckman model LS 3801 liquid scintillation counter (Beckman Instruments). The data presented reflect the typical results of at least three experiments.

Mapping

We measured co-transduction frequencies of zwittermicin A resistance loci and Tn*10* markers of known location on the *E. coli* chromosome with P1 *vir* generalized transducing phage (Singer *et al*., 1989). For initial mapping, P1 *vir* was grown on 24 separate mixtures of three or four Zm^s, Tn*10*-containing derivatives of strain MG1655 and, for further mapping, P1 *vir* was grown on individual Zm^s, Tn*10*-containing strains. Lysates were used to infect the Zm^r mutants, and tetracycline resistance, encoded by Tn*10*, was selected. Most transductants displayed the small colony morphology of the Zm^r mutant recipients. However, for each mutant recipient, at least one lysate yielded transductants with wild-type colony morphology. Between 60 and 600 transductants were scored for mutant or wild-type colony morphology and were considered Zm^r or Zm^s respectively. At least 12 transductants of each colony type were screened for zwittermicin A sensitivity, confirming that transductants with mutant and wild-type colony morphology were Zm^r and Zm^s respectively. Tn*10* markers that were not linked to Zm^r were included in each experiment as controls for spontaneous Zm^s revertants in the recipient culture. Tc^r Zm^r transductants were picked, colony purified and infected with P1 *vir* to generate lysates, which were used to infect MG1655. Tc^r transductants were selected, and the number of Zm^s and Zm^r transductants was estimated as above. P1 *vir* did not lyse Zm^r Tc^r derivatives of MGZ202, and Tc^r derivatives of MGZ203 were only lysed with *rpoC* provided on pRW308. Regions necessary for Zm^r and mutant colony morphology in mutants MGZ201, MGZ202 and MGZ203 were more precisely mapped by marker exchange. Zm^r mutants were transformed with plasmids bearing portions of *rpoC* (Heisler *et al*., 1996; this study), and eight colonies from each transformation were picked and streaked. Most transformed cells retained the slow growth phenotype of the host strain. However, in each mutant, certain plasmids gave rise to fast-growing colonies in all eight streaks, which were subsequently shown to be Zm^s.

Growth requirements of mutants

Mutants were streaked on 56 medium containing 30 mM glucose, succinate, malate or glycerol, and potassium nitrate for anaerobic respiration. Plates were incubated for 3 days at 37°C, and the appearance of colonies was scored as 'growth'. The wild-type strain MG1655 was a positive control and grew under all conditions.

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