IN VITRO DEVELOPMENT OF RESISTANCE TO FOSFOMYCIN

EUGENE L. DULANEY and CAROLYN L. RUBY

Basic Microbiology Research Department, Merck Institute for Therapeutic Research
Rahway, New Jersey, 07065, U.S.A.

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Resistant colonies develop with an apparent high frequency in zones of inhibition around fosfomycin filter discs on nutrient agar. The resistance is due, primarily, to loss of the fosfomycin transport system. The resistant colony type usually observed in the inhibition zones seldom arise directly by mutation from a cell sown in the area of the zone of inhibition. Instead, small translucent colonies develop first in the inhibition zone. Within these small translucent colonies, mutational events occur which give rise to the normal resistant type colonies.

Fosfomycin, (−)*cis*-1,2-epoxypropylphosphonic acid, is a broad spectrum[2] antibiotic produced by *Streptomyces* sp.[5] It has a high therapeutic index[4] and is used in treatment of human infections in Spain, Portugal and South America.

An interesting observation is that numerous resistant colonies appear in the inhibition zones during sensitivity testing to fosfomycin. The frequency with which the resistant colonies occur depends on the test bacterium but is most dependent on the assay medium. The present communication is directed to this problem of *in vitro* resistance development.

Experimental

Selection of Experimental Cultures

Representatives of *Escherichia* and *Salmonella* readily develop *in vitro* resistance to fosfomycin. Isolates of these genera were grown to late log phase in nutrient broth with agitation at 37°C, diluted in fresh broth and 0.1 ml containing 10^6 cells were spread over plates of nutrient agar. Filter discs containing fosfomycin were placed on the seeded surfaces and the plates incubated at 37°C. After 24 hours, there were numerous colonies in the zones of inhibition. *Escherichia coli* MSDRL 2017, and *Salmonella typhimurium* MSDRL 2637, were selected as the best for our experimental purposes. Hereafter, they will be referred to only with the strain numbers. It should be emphasized that these two species were selected for the ease with which they developed resistance to fosfomycin. Thus, they may not be complete representatives of the two genera.

Homogeneity of Experimental Cultures

There was the possibility that the fosfomycin resisters represented contaminants of the experimental cultures or resisters to fosfomycin that had arisen in the past in the absence of the drug. The fact that so many different isolates of various genera develop *in vitro* resistance would make these possibilities remote, nevertheless some experiments were directed to these points.

The test bacteria, 2017 and 2637, were grown to late log phase in nutrient broth, diluted in fresh broth and spread over the surface of nutrient agar plates. Ten single colonies of each specie were transferred to nutrient broth, grown to late log phase, diluted in fresh broth and 10^6 cells spread over the surface of nutrient agar plates. Filter discs containing fosfomycin were placed on the surfaces and the plates incubated at 37°C. After 24 hours incubation, numbers of resisters appeared in the zones of inhibition. The appearance of the resisters in all of the progeny from the single colony isolates eliminates the possibilities of contaminants or prior developed resisters as the explanation for resisters in the inhibition zone.

Frequency of Resistance

Since it was obvious that the *in vitro* resistance, as seen in the colonies in the zones of inhibition, was occurring in presence of fosfomycin some explanation for the high frequency of resistance was sought.

A simple explanation is that mutations to fosfomycin resistance are occurring in presence of the drug and the resisters grow into colonies in the zones of inhibition. The frequency with which such possible mutations occurred was calculated as follows. Late log phase cells of 2017 and 2637 were diluted in fresh broth and 10^6 cells were spread over the surface of nutrient agar plates. Filter discs, dipped into fosfomycin concentration of 500 μg, were placed on the surfaces of the seeded plates which were incubated at 37°C for 20 hours. The number of resistant colonies in the zones of inhibition were counted and the number of cells originally spread in the areas of the zones of inhibition were calculated. From these numbers the number of cells that possibly mutated to fosfomycin resistance were determined. The data are summarized in Table 1.

Table 1. Apparent frequency of resistance development to fosfomycin

Test organism	% of cells in the zone of inhibition that became resistant to fosfomycin
2017	8.4
2637	0.75

Based on the number of cells (input cells) in the zone of inhibition the frequency of mutation to resistance is very high. Indeed the frequency of resistance is so high that random mutation to fosfomycin resistance may be an over-simplistic explanation of resistance development under these conditions.

Causes of High Frequency of Resistance

The apparent very high frequency of resistance development is interesting and perplexing. Some of the more obvious reasons were investigated.

1. One possible explanation is that the resisters, inducibly or non-inducibly, inactivate the drug. We examined this possibility by measuring possible rate of fosfomycin loss in broth cultures of resisters and wild types. The results excluded this explanation. The experiments were not pursued exhaustively, however, for concurrent experiments showed the resisters to exclude the drug. Moreover, HENDLIN *et al.*[2] also showed resistance was not inducible. All of the resisters we have examined are mutants which have lost the ability to transport fosfomycin. They will not grow with L-α-glycerophosphate as the sole source of carbon. Fosfomycin is transported into bacterial cells by the L-α-glycerophosphate system.[3]

2. Another possibility in part for the high frequency of resistance is that fosfomycin might be a very potent mutagen. This was investigated by the following set of experiments.

a. Possible Reversal of Auxotrophs to Prototrophs:

Late log phase cells of auxotrophs grown in nutrient broth were diluted in fresh broth and 10^6 added to two ml of nutrient broth containing fosfomycin concentration just below the MIC. After 20 hours incubation with shaking at 37°C, the cells were washed with the minimal medium of DAVIS and MINGIOLI[1], diluted in the same medium and plated, at high concentration onto the minimal agar medium. Auxotrophs were grown in the absence of fosfomycin and treated similarly.

The auxotroph tester strains had single requirements:

Species	Requirement	Species	Requirement
Bacillus subtilis	methionine	*Escherichia coli*	histidine
Escherichia coli	methionine	*Salmonella typhimurium*	arginine
Escherichia coli	tryptophan	*Salmonella typhimurium*	glutamic acid

There was no significant increase in numbers of prototrophs from any auxotroph grown in the presence of fosfomycin over the numbers from auxotrophs grown in the absence of fosfomycin. In a parallel experiment fosfomycin was tested for ability to revert a streptomycin dependent mutant of

Escherichia coli to streptomycin independence. The drug did not induce the reversion.

b. Possible Mutation to Fosfomycin Resistance:

More to the point is the possibility that fosfomycin is inducing mutations to fosfomycin resistance. Cultures 2017 and 2637 were grown to late log phase in nutrient broth, centrifuged and resuspended at a concentration of about 10^9 cells per ml in tubes of nutrient broth, some of which contained 1 μg/ml of fosfomycin, and some which contained no drug. After two hours incubation at 37°C, the growth were diluted and spread over plates of nutrient agar containing 25 μg/ml of fosfomycin and plates that contained no drug. Counts made after 24- and 48-hour incubation showed no increase in the number of fosfomycin resisters in cultures grown in presence of the drug over the number of resisters in cultures grown in absence of the drug.

c. Infective spread of resistance might also explain the high frequency of resistance development to fosfomycin. It is unlikely for resistance occurs in so many genera in the absence of clinical use of the drug. If the genetic determinants for fosfomycin resistance are so widespread on plasmids, they must confer some selective advantage other than fosfomycin resistance to the bacteria bearing the plasmids.

We investigated the possibility of R factor transfer of fosfomycin resistance in the following set of experiments. Ten fosfomycin-resistant strains from *E. coli* 2017 were grown to late log phase with agitation at 37°C in nutrient broth containing 100 μg/ml of fosfomycin. The recipient *E. coli* was resistant to nalidixic acid and furazolidone and was grown to late log phase in nutrient broth containing 10 μg/ml of nalidixic acid and 2 μg/ml of furazolidone. The cultures were diluted 1: 10 in fresh broth and incubated with shaking at 37°C for 3 hours. Three ml of each donor were mixed with 6 ml of the recipient in 250 ml Erlenmeyer flasks. The mating mixtures were incubated without agitation for 24 hours at 37°C, diluted in fresh broth, and plated on selective media. After 24- and 48-hour incubation, no colonies developed on the medium containing fosfomycin 100 μg/ml, nalidixic acid 10 μg/ml and furazolidone 2 μg/ml. This is the medium that would be selective for recipients that became fosfomycin resistant. Platings of donors, recipient and mating mixtures on other media showed them to behave as expected.

In a parallel experiment, fosfomycin resistant *Proteus morganii* and *Proteus mirabilis* served as the donor and were mated with the recipient *E. coli* used above. In this experiment E. M. B. agar containing nalidixic acid, furazolidone and fosfomycin was used as the medium for plating the mating mixtures. No fosfomycin resistance was transferred to the recipient.

Mechanism of Resistance

The mechanism of fosfomycin transport has been elucidated by KAHAN, CASSIDY and collaborators.[3] Fosfomycin is transported into bacterial cells mainly by the L-α-glycerophosphate system and acts by inhibition of phosphoenolpyruvate: UDPGlcNac-3-enolpyruvate transferase. The resisters that occur in zones of inhibition around fosfomycin containing filter discs have lost the above transport system and cannot take in fosfomycin. They will not grow with L-α-glycerophosphate as the sole source of carbon. The loss of this transport system is a stable genetic change. The loss is not caused by fosfomycin induced mutation and the determinants are not borne on a transferable plasmid. Sensitivity to fosfomycin cannot be regained by growth of the resisters in absence of fosfomycin. The resisters do not degrade fosfomycin.

Appearance of resisters in the zone of inhibition can be eliminated by using DAVIS and MINGIOLI minimal medium[1] instead of nutrient agar as the assay medium or by addition of glucose-6-phosphate to the nutrient agar. The addition of glucose-6-phosphate induces the hexose phosphate uptake system by which fosfomycin can enter bacterial cells.[3] Thus L-α-glycerophosphate transport negative resisters can be killed by fosfomycin entering by the induced hexose phosphate transport system. Still the apparent high frequency of resisters, due to loss of the L-α-glycerophosphate transport system, that develop in the zones of inhibition around filter discs of fosfomycin on nutrient agar remains unexplained.

Homogeneity of Resistant Colonies

Resistant colonies of 2017 and 2637 were cut from a zone of inhibition, transferred to nutrient broth, mixed vigorously on a vortex mixer for one minute, diluted in fresh broth, and spread over plates of nutrient agar containing concentrations of fosfomycin. The results are summarized in Table 2. Clearly, the colony-forming units within these resistant colonies are not uniformly resistant to fosfomycin. Different levels of resistance may occur in increments rather than in one step. This was proven by the following replicate plating experiment.

Six isolated colonies of 2017 and 2637 were transferred to nutrient agar plates and spread over an area of approximately 1 cm^2. After the colonies developed, they were replicated, using velveteen, to nutrient agar plates containing increasing concentration of fosfomycin. Resistant colonies that developed on plates containing high, but subinhibitory concentrations of fosfomycin, were replicated onto nutrient agar plates containing even higher concentrations of fosfomycin. This experiment was continued through three replications. The plates were always incubated at 37°C for 24 hours. Since similar results were obtained with both cultures only the replications with 2637 are shown in Fig. 1. Rather than show replication of all colonies, enlarged single colonies are presented. The lack of homogeneity of resistance in the colonies is clear as is the isolated occurrence of resistant areas in the colonies which on subsequent replication show increasing levels of resistance.

Table 2. Levels of fosfomycin resistance in cells of resistant colonies

Concentrations of fosfomycin—μg/ml in plating agar	No. of cells in resistant colonies of	
	2017	2637
0	1.81×10^6	2.28×10^7
5	1.7×10^6	1.55×10^4
10	1.7×10^5	1.15×10^4
20	3.6×10^4	3.0×10^3
50	3.0×10^1	2.0×10^3
100	$<10^1$	$<10^1$

10^6 Cells of 2017, grown in absence of fosfomycin, contained 1.2 which would grow when plated onto agar containing 20 μg/ml of fosfomycin. Two of 10^6 cells of 2637, grown in absence of fosfomycin, would grow on agar containing 5 μg/ml of drug.

Development of Resistant Colonies

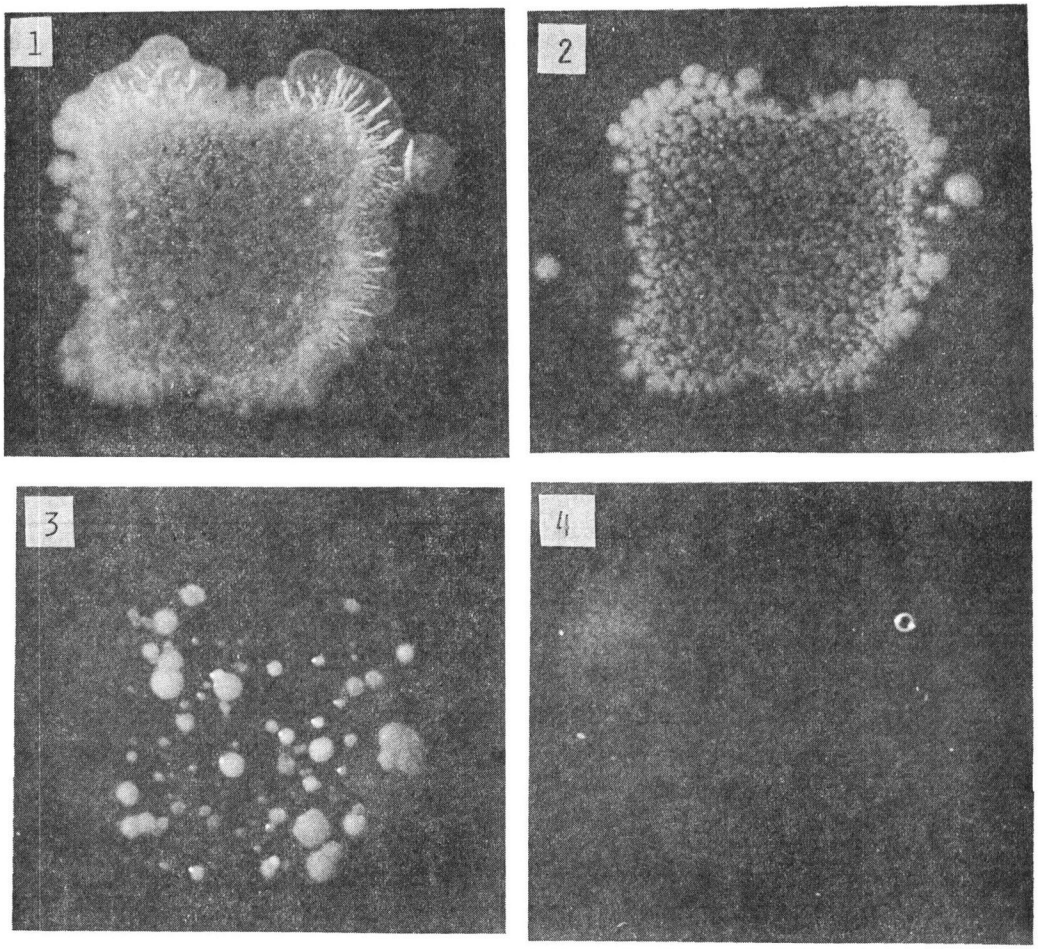
Close observation using low power optics of the zones of inhibition around discs of fosfomycin on nutrient agar spread with 2017 and 2637 revealed the means by which resisters were developing as well as the explanation for the apparent high frequency of resistance. On these plates, two types of colonies were visible; (1) the normal appearing resister (N) and (2) smaller transluscent colonies (STC).

Table 3. Relation of fosfomycin concentration to appearance of N and STC types of resisters.

Fosfomycin μg/ml	CFU of			
	2017		2637	
	N	STC	N	STC
0	1.81×10^6	0	2.28×10^7	0
5	1.7×10^6	7.9×10^5	1.55×10^4	2.43×10^5
10	1.7×10^6	2.3×10^6	1.15×10^4	4.0×10^4
20	3.6×10^4	1.1×10^6	3.0×10^3	1.2×10^4
50	3.0×10^1	5.7×10^2	2.0×10^3	0
100	0	0	0	0

Fig. 1. Replication of colonies of 2637 onto medium containing increasing concentrations of fosfomycin. A colony growing on medium with no fosfomycin was replicated onto medium containing:

No. 1. No Fosfomycin
No. 2. 50 μg/ml Fosfomycin
No. 3. 100 μg/ml Fosfomycin
No. 4. 250 μg/ml Fosfomycin



The resistant colonies growing on medium containing 100 μg/ml of fosfomycin, No. 3 in this figure, were replicated onto medium containing:

No. 5. 100 μg/ml Fosfomycin
No. 6. 200 μg/ml Fosfomycin

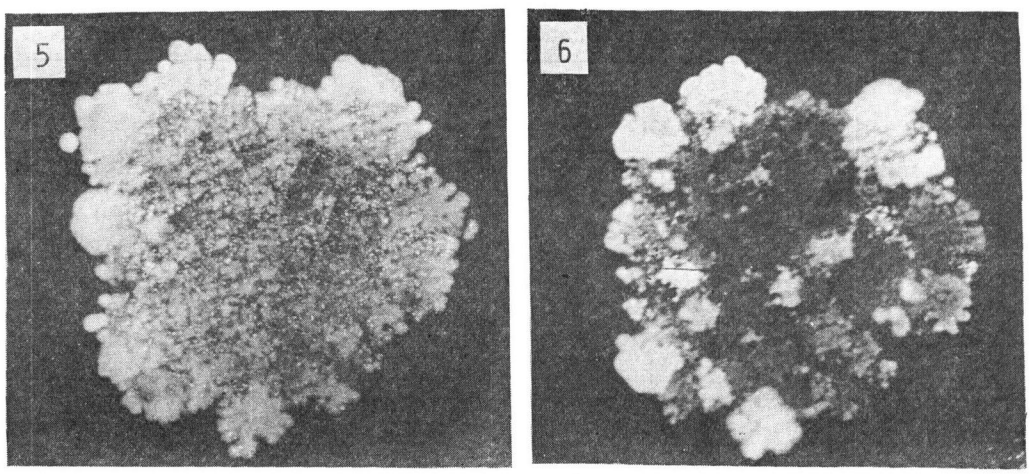
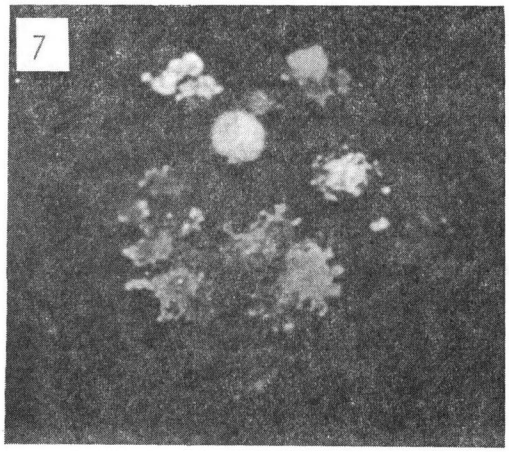
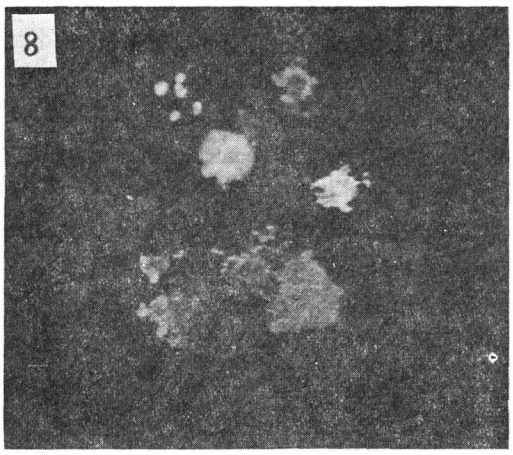


Fig. 1. Continued

No. 7. 300 μg/ml Fosfomycin

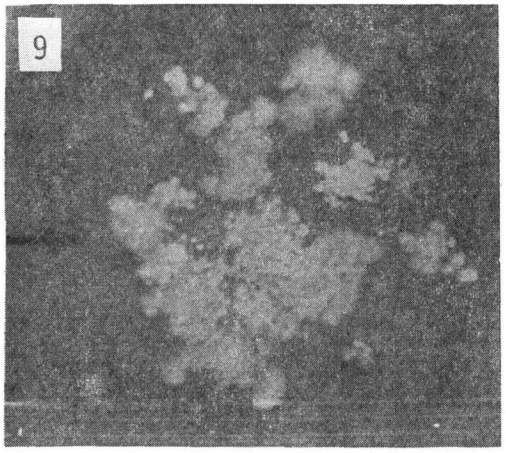


No. 8. 400 μg/ml Fosfomycin

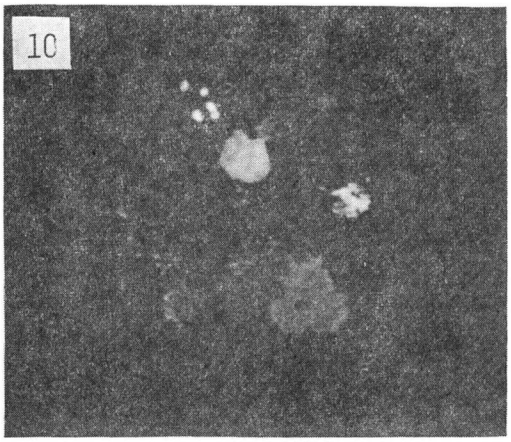


The resistant colonies growing on medium containing 400 μg/ml fosfomycin, No. 8 in this figure, were replicated onto medium containing:

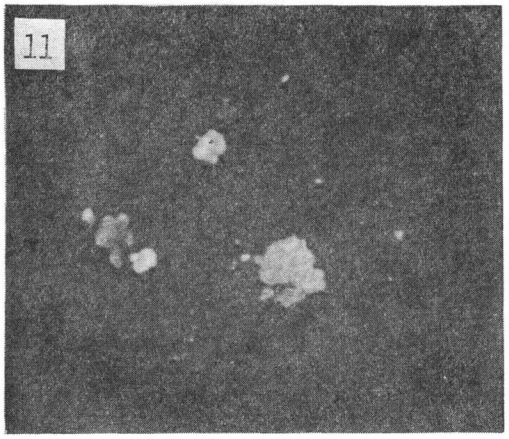
No. 9. 500 μg/ml Fosfomycin



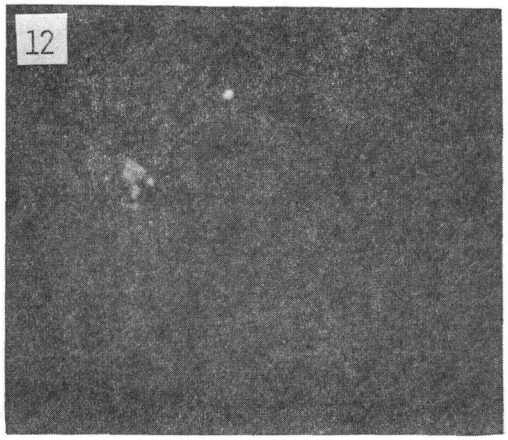
No. 10. 600 μg/ml Fosfomycin



No. 11. 800 μg/ml Fosfomycin



No. 12. 1000 μg/ml Fosfomycin



The STC never appear in the absence of fosfomycin. The significance of the STC in development of resisters to fosfomycin was investigated by the following series of experiments.

1. Relation of Fosfomycin Concentration to the Two Colony Types

A normal appearing (N) resistant colony of 2637 and 2017 was cut from a zone of inhibition, each vortexed in nutrient broth, diluted in nutrient broth and spread over the surface of nutrient agar containing increasing concentrations of fosfomycin. The results are in Table 3. Clearly, there is a marked decrease of N types and a marked increase of STC types with increasing concentrations of fosfomycin.

2. Cell Types in STC

STC types of 2017 and 2637 were transferred to nutrient broth containing 10% sucrose and examined at a magnification of 1,000× using phase optics. A gradation of cell types from typical L forms to normal bacilli were observed. While we have refrained from calling the STC types L forms or protoplast colonies, it is clear that the STC resemble L form colonies in some respects, particularly in the presence of a large frequency of protoplasts or at least forms with little or aberrant walls. STC's appear only in the presence of fosfomycin, a drug known to interfere with cell wall synthesis. Moreover, we have no evidence that the STC type represents a permanent change.

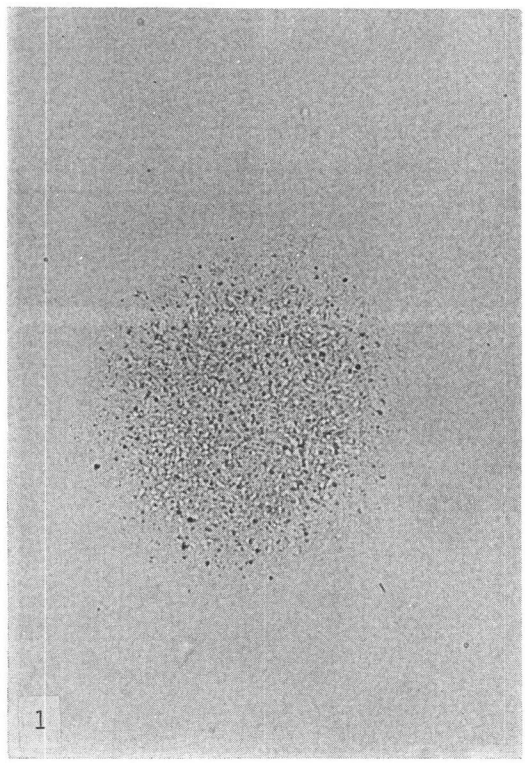
3. Appearance of N Resisters in STC Types

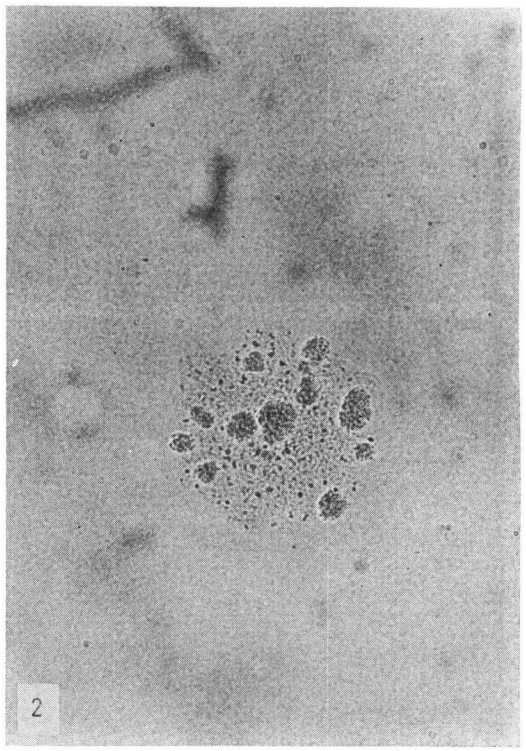
Observation of resisters developing in the zones of inhibition showed that most N colonies were developing from STC's. They can be seen first as papillae or denser points of growth that grow into N types. This is shown in Fig. 2. Clearly, some cells respond to fosfomycin by first growing into

Fig. 2.

1. Small transluscent colony (STC) 50 with no resistant papillae.

2. Small transluscent colony (STC) 50 with numerous resistant papillae.





STC's. Some osmotic stabilization would probably be required for this and perhaps nutrient agar is sufficient to so stabilize the cells of the STC's. Within one or more cells of the STC's an event occurs which results in fosfomycin resistance and the N colonies grow out as a result. Thus, the large frequency of resisters based on numbers of in-put cells in the zone of inhibition (Table 1) is an invalid estimation of the frequency of mutation to fosfomycin resistance. The N colonies are arising not from a single isolated cell in a zone of inhibition, but from the population of cells in developing STC's.

One can estimate the frequency at which the N type colonies arise per colony-forming-unit (CFU) in the STC's. The number of papillae occurring in a large number of STC's from 2017 and 2637 were scored using low power optics. The average number of CFU per STC was determined for 2017 and 2637 by cutting out 20 STC's of each, which contained no papillae, vortexing them in nutrient broth and plating on nutrient agar. Counts were made after 24 hours incubation at 37°C. The data are summarized in Table 4.

Table 4. Appearance of papillae types in STC types.

	2017	2637	
	24 hours	24 hours	48 hours
Average No. of CFU per STC	5.44×10^5	4.21×10^4	8.9×10^5
No. of STC's scored	181	289	263
No. of papillae	18	41	584
Average No. of papillae types per STC	0.1	0.142	2.2
Frequency of papillae types per CFU in STC	1 per 5.5×10^6	1 per 4.2×10^5	1 per 4.4×10^5

The frequencies with which the N types occur per CFU in STC's, reported in Table 4, should be taken as a minimum figure. The stability of cells in the STC's to the procedures used in making the CFU counts is not known. Since many of them may be fragile because of absent, reduced or aberrant cell walls, the counts of CFU may be much lower than the actual number of viable cells in the STC's.

Conversely one might question whether the number of papillae scored after 24 hours incubation is low. This could be possible if the events leading to papillae occurred too late for sufficient cell division to permit the emerging papillae to reach a size large enough to be seen and scored. This possibility is unlikely for there was no increase in frequency of papillus per CFU in 2637 from 24 to 48 hours. With further incubation the papillae grow together and prevent further scoring. In 2017, the papillae grow more rapidly than with 2637 and may have merged between 24 and 48 hours. Since we were not sure of these data they are not entered in Table 4.

If the event, presumably a mutation, that results in fosfomycin resistance occurs independently, then the number of events reflected as N colonies developing in STC's should fit a POISSON distribution. This was investigated by spreading cells of 2017 and 2637 diluted in nutrient broth over plates of nutrient agar containing fosfomycin, 9 μg/ml for 2017 and 0.9 μg/ml for 2637. After 24 hour-incubation, the number of emerging N types were counted in the STC's. The data are in Tables 5 and 6. The observed frequency of events in the STC's that result in resistance does not fit the POISSON expected frequency. We have not presented the frequency of emerging N types after 48 hour-incubation but it is still far from the POISSON expected frequency. Thus, the event leading to the formation of N types does not occur at random or its expression is affected by something else.

Table 5. Frequency distribution of papillae in STC's of 2017 and the expected frequency for the POISSON distribution

Number of events occurring in a colony	Observed frequency	POISSON expected frequency
0	101	110.85
1	163	127.90
2	46	73.50
3	23	28.22
4	11	8.13
5	5	1.95
6	2	0.45
Chi-square	—	32.89
D.O.F.		5
Significance level, P.		<0.0001

Table 6. Frequency distribution of papillae in STC's of 2637 and the expected frequency for the POISSON distribution

Number of events occurring in a colony	Observed frequency	POISSON expected frequency
0	70	45.7
1	73	82.2
2	41	71.6
3	38	39.7
4	28	18.6
5	10	5.3
6	3	1.6
7	2	0.3
Chi-square		46.88
D.O.F.		6
Significance level, P		<0.001

Discussion

The reason for the apparent high frequency of resistance in some genera to fosfomycin now seems clear. Some cells in the population are able to grow with aberrant or little cell wall into colonies which we have chosen to call small translucent colonies (STC's). Some cells in the STC's then mutate to resistance of a type that allows them to generate cell wall and emerge as normal colonies. These resisters are of the glpT-type, that is they lack the L-α-glycerophosphate transport system. These will not grow on α-glycerophosphate as the sole carbon source and they disappear when the hexose-phosphate (uhp) transport system is induced. Fosfomycin is transported by this system and thus kills the glpT-resisters that would develop.

Thus, based on the number of papillae per CFU in the STC's, the frequency of *in vitro* resistance development to fosfomycin is not particularly high. Two caveats discussed above must be re-emphasized, however. The STC's could well contain osmotically fragile cells which would not develop into colonies when plated out resulting in a low count of CFU's and a higher calculated frequency of resistance. Conversely, it is possible that all mutations to resistance would not be scored if the emerging papillae were too small to be visible at the time counts were made. Also, developing papillae could escape counting if they merged with adjacent larger papillae. These possibilities would lead to a lower calculated frequency of resistance and would be most likely to occur with 2017 STC's in which papillae develop rapidly. The fact that resistance occurs stepwise should not effect these results since the fosfomycin concentration used in these determinations fall well within the first resistance step.

The relationship of our N and STC types to the colony types reported by TSURUOKA and YAMADA[6] is not clear.

Acknowledgement

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