

Transmissible drug resistance in an epidemic strain of *Salmonella typhimurium*

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INTRODUCTION

During an outbreak of infection in a general hospital in London in 1959, 252 people were found to be excreting *Salmonella typhimurium* (Datta & Pridie, 1960). At that time 325 of the cultures isolated were put aside for further study. These cultures originated as single non-lactose-fermenting colonies on the media used diagnostically, i.e. MacConkey agar or deoxycholate-citrate agar, which had been subcultured and identified by fermentative and serological reactions as *S. typhimurium*. They were subcultured again on Dorset egg slopes in screw-capped bottles and kept at room temperature. Some of them were at the time of isolation sent to the Enteric Reference Laboratory, Colindale, where they were found to belong to phage-type 27 (see Callow, 1959).

In the latter part of 1961, in the course of investigating these cultures for the homogeneity of some of their properties, their sensitivity to various antibiotics was tested. The results of the sensitivity tests were of sufficient interest to warrant further investigation. More detailed experiments were made, some of which are recorded in the following account.

Sensitivity tests

The drugs used were ampicillin, streptomycin, tetracycline, chloramphenicol and sulphathiazole.

Sensitivity was tested on nutrient agar ditch plates for the four antibiotics, and on laked horse-blood plates (Harper & Cawston, 1945) for sulphathiazole. The concentrations used are listed in Table 1.

Each culture was streaked directly from the stored slope across each ditch plate, and was spread on the sulphathiazole plate in such a way as to obtain single colonies. At the same time the cultures were plated on nutrient agar to exclude obvious contamination, and also for other tests not reported here.

There were 309 cultures tested for sensitivity. Of the others, two were contaminated with proteus and the salmonella could not be recovered; in fourteen the medium had dried up due to faulty fitting of the screw-caps, and the cultures were dead.

For 309 cultures tested, the results are shown in Table 2.

Strains resistant to a single drug

Of the eight streptomycin-resistant (strep^r) strains, five came from one patient, two from another and one from a third. All these three patients had been treated with streptomycin. The two patients from whom tetracycline-resistant and sulphathiazole-resistant strains were isolated had been treated with tetracycline and sulphonamides respectively. The earliest isolates of *S. typhimurium* from four of these five patients were fully sensitive to all the drugs tested.

Table 1

Drug	Abbreviation	Concentration in ditch ($\mu\text{g./ml.}$)
Ampicillin	Amp.	50
Streptomycin	Strep.	25
Tetracycline	Tet.	25
Chloramphenicol	Chlor.	25
		Concentration in plate ($\mu\text{g./ml.}$)
Sulphathiazole	Sulph.	100

Table 2. *Distribution of drug-resistance in the Salmonella typhimurium cultures*

Sensitive to all 5 drugs	Resistant only to:			Resistant to strep., tet., and sulph.
	Strep.	Tet.	Sulph.	
284	8	1	1	15

Resistance was recorded when the culture grew uninterruptedly across the antibiotic ditch or grew as single colonies on the sulphathiazole plate.

Sensitive cultures stopped growing short of the ditch, but grew rather nearer to it than did the Oxford staphylococcus. They failed to form colonies on the sulphathiazole plates.

Strains resistant to three drugs (r₃)

The fifteen strains resistant to streptomycin, tetracycline and sulphathiazole were isolated from eight patients, none of whom had been treated with all three of these drugs. In most of these cases, too, the first isolates of *S. typhimurium* were fully drug-sensitive. These eight patients came from seven different wards and the two who were in the same ward were not there at the same time.

Information on these eight patients is given in Table 3.

It can be seen from Table 3 that with one exception reversion to sensitivity did not occur. Patients from whom triple-resistant organisms were isolated continued to excrete such organisms as long as their stools remained positive. The one exception is patient no. 5, from whom, on 1 July 1959, both a sensitive and a triple-resistant isolate is recorded. This was in fact only one culture, made in the same way as the others, but on the antibiotic ditch plates it could be seen to consist of a mixture of sensitive and resistant organisms.

Table 3 shows that in most instances resistance to all three drugs appeared simultaneously, but in one instance (patient no. 4), resistance to tetracycline

appeared first. This was the sole instance of a culture resistant only to tetracycline and is recorded also in Table 2. It was isolated from the patient on the sixth day of treatment with tetracycline. This culture will be referred to again (*S. typhimurium* 27 tet^r).

Table 3. *Patients from whom multiple-resistant Salmonella typhimurium was isolated in 1959*

Patient	Ward	Treatment	Date of isolation of <i>S. typhimurium</i>		
			Fully sensitive	Resistant to tet.	Resistant to strep., tet., and sulph.
1	B 5	18/4, strep. + neo.	15/4	None	20/4
2	B 1	29/4 tet. + chlor.	29/4	„	6/5, 8/5, 9/5
3	H 5	15/5, strep. + sulph.	11/5	„	25/5, 27/5, 30/5, 1/6
4	B 6	11/4, strep. + neo. 4/6, tet. 23/6, strep.	4/5, 6/5, 9/5	10/6	1/7
5	PBU	14/6, strep. + sulph. + chlor. 1/7, neo.	14/6, 15/6, 26/6, 29/6, 1/7, 2/7, 7/7, 14/7, 4/8	None	1/7
6	H 3	14/4, sulph. + neo. 22/5, neo	None	„	19/5
7	B 1	22/5, strep. 29/5, chlor. + neo.	None	„	20/5, 25/5
8	D 6	None	None	„	23/5

neo. = neomycin

This table records all antibacterial drugs administered to the patients at the relevant time. The dates are those on which the first dose was given. In most instances the drugs were continued for 5 days. Some of these drugs were prescribed because of the salmonella infection, and some for unrelated illness.

Phage-typing

All the resistant strains, together with sensitive strains isolated from the same patients, were sent to the Enteric Reference Laboratory for phage-typing. All were of phage type 27. (Some had already been typed in 1959, but were sent again in 1962).

EXPERIMENTAL

Transfer of resistance

Because of reports from Japan (Watanabe & Fukasawa, 1961*a*) of transfer of multiple antibiotic resistance between species of enteric bacteria, experiments were made to find whether the resistance of these cultures of *S. typhimurium* was transferable.

Method

(1) Single colonies of the following organisms were inoculated into broth and incubated overnight at 37° C.: *Salmonella typhimurium* type 27 triply-resistant (*S. typhimurium* 27_{r₃}), and *Shigella sonnei* 538 (a recently isolated strain sensitive to all antibiotics tested, received from Dr K. P. Carpenter, Shigella Reference Laboratory, Colindale).

(2) A mixed culture was made, 0.02 ml. of *S. typhimurium* 27_{r₃} and 0.5 ml. of *S. sonnei* 538 were added to 5 ml. broth, incubated at 37° C. overnight, then diluted with 50 ml. of fresh, warm broth and incubated again overnight.

This method was used because it has been found by Stocker and Smith (Stocker, 1960) to be effective in the transfer of colicinogenicity in *S. typhimurium*.

(3) Dilutions of the cultures were spread on plates of MacConkey agar, with and without the addition of streptomycin and tetracycline (25 µg./ml. of each) and incubated overnight.

(4) The plates were examined and differential counts of salmonella and shigella made according to colonial appearance.

Note. In making differential counts in this way it was found that the shigella count was always relatively lower in the low dilutions than in the high dilutions, i.e. it was lower when the total count was high. This was found (by picking all colonies from a marked area of plate for serological identification) to be due to the fact that only the rougher shigella colonies could be identified morphologically when the plate was crowded.

Table 4. *Results of transfer experiment by growth in mixed culture of Salmonella typhimurium 27 r₃ and Shigella sonnei 538*

Dilution of mixed culture (0.5 ml. plated)	Colony counts on:			
	MacConkey		MacConkey + strep. + tet.	
	<i>Salmonella</i>	<i>Shigella</i>	<i>Salmonella</i>	<i>Shigella</i>
10 ⁻⁶	> 100	> 100	> 100	5
10 ⁻⁷	62	41	71	1
10 ⁻⁸	9	13	6	0

No growth was observed when *Sh. sonnei* 538, grown in parallel but in the absence of *S. typhimurium*, was plated on MacConkey + strep. + tet.

Results

The differential colony counts of this experiment are shown in Table 4. It can be seen that six shigella colonies were present on the antibiotic plates. These were purified on nutrient agar plates. Their fermentative and serological reactions were typical of *Sh. sonnei* and they were resistant to streptomycin, tetracycline and sulphathiazole (r₃) but sensitive to the other antibiotics tested. Transfer had occurred, approximately 2% of the sensitive *Sh. sonnei* culture having acquired triple drug-resistance. This experiment was repeated many times and always between 0.5 and 5% of the *Sh. sonnei* became resistant. No resistant colonies were ever obtained in pure, control cultures of *Sh. sonnei* 538.

Transfer of resistance back to a sensitive culture of S. typhimurium 27

Sh. sonnei 538 r₃ was grown in mixed broth culture as described above with a sensitive strain of *S. typhimurium* 27 isolated during the 1959 epidemic.

When the mixed culture was plated on MacConkey agar as described above, no resistant salmonella colonies were seen. The organisms in 5 ml. of the mixed culture were washed three times with saline, resuspended in 0.5 ml. saline, and inoculated on to minimal agar (Davis & Mingioli, 1950) incorporating streptomycin and tetracycline. This medium would not support the growth of *Sh. sonnei* because of its requirement for nicotinic acid.

Table 5. *Shigella sonnei* 538 grown in sterilized culture medium from *Salmonella typhimurium* 27 r₃

Dilution of culture (0.5 ml. plated)	Colony counts on			
	MacConkey		MacConkey + strep. + tet.	
	<i>Salmonella</i>	<i>Shigella</i>	<i>Salmonella</i>	<i>Shigella</i>
Heated medium				
10 ⁻⁶	.	.	0	0
10 ⁻⁷	0	> 100	0	0
10 ⁻⁸	0	63	0	0
Filtered medium				
10 ⁻⁶	.	.	0	0
10 ⁻⁷	0	61	0	0
10 ⁻⁸	0	9	0	0

From an inoculum consisting of about 10¹⁰ cells of *Sh. sonnei* 538 r₃ and 10¹⁰ cells of *S. typhimurium* 27, thirteen colonies developed on antibiotic minimal agar. On purification they proved to be *S. typhimurium* 27, resistant to streptomycin, tetracycline and sulphathiazole (r₃). Transfer had occurred. About 1:10⁹ of the previously sensitive salmonellae had acquired triple drug-resistance.

Attempted transfer of resistance by cell-free culture medium

S. typhimurium 27 r₃ was grown in pure culture in broth, then diluted 10 times with fresh broth and re-incubated as described above. The culture was then centrifuged and the supernatant fluid divided into two equal parts. One part was heated at 56° for 45 min., the other was filtered through a membrane filter (average pore diameter 0.45 μ). One ml. of each was inoculated into broth as a sterility test.

Sh. sonnei 538, 0.5 ml. of an 18 hr. broth culture, was added to each part of the sterilized medium and incubated overnight. Growth was poor, but further incubation on a shaker for 2 hr. gave good turbidity. The broths were then diluted and plated on MacConkey agar with and without antibiotics. The results are shown in Table 5. No transfer of resistance was demonstrated.

Summary of transfer experiments

The results of other transfer experiments using the methods described above are summarized in Table 6. It will be seen that the tetracycline-resistant culture of

S. typhimurium 27 transmitted its drug-resistance to *Sh. sonnei*, but that a streptomycin-resistant culture did not.

Levels of resistance

The levels of antibiotic resistance in these cultures were measured by recording the growth of a small inoculum (approximately 2000 organisms) in serial dilutions of the antibiotic in broth, and levels of sulphonamide resistance by plating out on laked blood plates incorporating serial dilutions of sulphathiazole. The results of these experiments are shown in Table 7.

Table 6. *Summary of transfer experiments*

Resistant culture	Sensitive culture	Occurrence of transfer	Approximate proportion of sensitive culture rendered resistant
<i>S. typhimurium</i> 27 r ₃	<i>Sh. sonnei</i> 538	+	10 ⁻²
	<i>Sh. sonnei</i> 487 (new isolate)	-	.
	<i>E. coli</i> (new isolate)	-	.
	<i>E. coli</i> K 12 F +	-	.
	<i>E. coli</i> K 12 F -	-	.
<i>Sh. sonnei</i> 538 r ₃	<i>S. typhimurium</i> 27	+	10 ⁻⁹
	<i>S. typhimurium</i> LT 2	+	10 ⁻⁹
	<i>E. coli</i> K 12 F +	-	.
	<i>E. coli</i> K 12 F -	-	.
<i>S. typhimurium</i> 27 strep ^r	<i>Sh. sonnei</i> 538	-	.
<i>S. typhimurium</i> 27 tet ^r	<i>Sh. sonnei</i> 538	+	10 ⁻²

Table 7. *Levels of drug resistance*

Culture	Growth inhibited by (µg./ml. medium)		
	Strep.	Tet.	Sulph.
<i>S. typhimurium</i> 27 (sensitive)	20	1	10
<i>S. typhimurium</i> 27 strep ^r	> 1000	1	10
<i>S. typhimurium</i> 27 tet ^r	20	400	10
<i>S. typhimurium</i> 27 r ₃	1000	400	> 4000
<i>Sh. sonnei</i> 538 (sensitive)	10	< 1	20
<i>Sh. sonnei</i> 538 r ₃	1000	400	> 4000

Stability of resistance

In serial culture, in broth or on solid media, the drug-resistance of these cultures appeared to be quite stable. It has been suggested that an antibiotic resistance factor can be carried on an episome, and be eliminated by treatment with acriflavine (Watanabe & Fukasawa, 1961*b*; Mitsuhashi, Harada & Kameda, 1961). *S. typhimurium* 27 r₃ was cultured at 37° C. overnight in broth with 100 µg./ml. acriflavine, a concentration which allowed suboptimal growth. A control culture was grown in broth without acriflavine. Both cultures were spread on nutrient

agar plates at dilutions which gave about 50 colonies per plate, and incubated overnight. The colonies were then inoculated on to plates containing streptomycin (25 $\mu\text{g./ml.}$), tetracycline (25 $\mu\text{g./ml.}$) or sulphathiazole (100 $\mu\text{g./ml.}$) by velvet-pad replica plating (Lederberg & Lederberg, 1952). It was found that this method failed to detect colonies sensitive to tetracycline or sulphathiazole. Sensitive replicated colonies grew quite well on plates containing these drugs, even when their concentration was increased tenfold (to 250 and 1000 $\mu\text{g./ml.}$, respectively), presumably because the inoculum was too large.

On comparing the master plates with the streptomycin-containing plates, however, a few colonies were found to be absent on the latter. When these were picked from the master plates and tested, they were found to be sensitive to streptomycin, tetracycline and sulphathiazole. The proportion of sensitive colonies was about the same in the acriflavine-treated as in the control cultures:

Culture	Total no. colonies tested	No. sensitive colonies	Percentage sensitive colonies
Acriflavine-treated	467	2	0.43
Control, untreated	1067	6	0.56

Selection of resistant variants in sensitive cultures

Antibiotic resistance was sought in the sensitive strains of *S. typhimurium* 27 from the 1959 outbreak. Cultures were grown in 100 ml. volumes of broth on a shaker for 5 hr., then diluted with an equal volume of broth containing streptomycin 500 $\mu\text{g./ml.}$ After overnight incubation the cultures were plated on streptomycin-agar plates. Numerous colonies developed which were found to be resistant to streptomycin but sensitive to tetracycline and to sulphathiazole. Attempts to isolate organisms resistant to both streptomycin and tetracycline by screening the streptomycin-resistant cultures with tetracycline have so far failed.

Resistance of enteric bacteria other than Salmonella typhimurium 27

Twenty-one strains of various enteric bacteria other than *S. typhimurium* 27, isolated from patients in the hospital in 1959, and 120 isolated in 1962 were tested for drug-sensitivity. The 1959 strains were mostly pathogens (18 salmonellae, 2 escherichiae, and 1 shigella) and the 1962 strains were normal bowel bacteria (*Escherichia coli*). Resistance to streptomycin, tetracycline and sulphathiazole was found in one culture of *Sh. sonnei* isolated in 1959 and in three of the *E. coli* isolated in 1962. Attempts to transfer resistance from these organisms to other bacteria by growth in mixed culture failed and no sensitive variants were found by the replica-plating technique, with or without previous exposure to acriflavine.

DISCUSSION

The cultures of *S. typhimurium* studied could reasonably be considered to have a common origin, since all were isolated in one outbreak of human infection and all were of the same phage type. The distribution of drug-resistant strains among them (see Table 2) was therefore very surprising. In such a collection of cultures,

a few strains resistant to one or other antibacterial drugs being used therapeutically could be expected on the basis of the selection of occasional resistant mutants. Even strains resistant to more than one drug might have been explained in the same way; resistance to each drug could have been selected successively during the course of an epidemic which lasted for several months (Datta & Pridie, 1960).

The finding, however, that there were more strains resistant to three drugs simultaneously than there were strains resistant to only one drug, while none was resistant to only two drugs, indicated that some other explanation was required. If the chromosomal loci controlling resistance to streptomycin, tetracycline and sulphonamide are closely linked in *S. typhimurium* a single mutation might induce simultaneous resistance to all three drugs. Such a mutant would be selected by treatment with any one of the three. Seven of the eight patients who excreted triple-resistant salmonellae were treated with one or more of the three drugs (see Table 3) and from the eighth, the only salmonella isolated was drug-resistant and she may have been primarily infected with a resistant strain.

There is no other evidence, however, that resistance to the three drugs is closely linked genetically; there is some evidence that it is not. Variants selected for streptomycin resistance were never found to be resistant to tetracycline or sulphathiazole, and triple-resistant variants never developed in the control cultures of the transfer experiments described in this paper, although some would have been expected if triple-resistance were the result of a single mutation.

It seems more probable that this drug resistance is analogous to that reported from Japan, and first encountered there in *Shigella flexneri*. Japanese workers (Watanabe & Fukasawa, 1961*a, b, c*; Mitsuhashi, Harada & Hashimoto, 1960; Harada, Suzuki, Kameda & Mitsuhashi, 1960; Mitsuhashi, Harada, Kameda, Suzuki & Egawa, 1960; Mitsuhashi, Harada, Hashimoto & Egawa, 1961*a, b*; Mitsuhashi, Harada & Kameda, 1961) have reported that a factor or factors controlling multiple drug resistance can be transferred at cell conjugation between Gram-negative bacilli of many different species. They have presented evidence that the factors responsible are carried on an episome, as defined by Jacob & Wollman (1958); that is, a genetic element which may be present in the cell or absent from it and which when present may either replicate autonomously, not necessarily at the same rate as the cell nucleus, or may be integrated into the chromosome and be replicated with it.

The triple drug resistance in *S. typhimurium* described in this paper resembles that described in Japan in that it can be transferred to another bacterial species in mixed culture. It is likely that transfer is by cell conjugation, since no resistance developed in sensitive organisms grown in cell-free culture medium of the resistant strain. But for this, it might have been considered that the resistance transfer was an example of transduction, since all the *S. typhimurium* 27 cultures were lysogenic and carried a heat-stable type A bacteriophage (Boyd & Bidwell, 1957).

The triple drug resistance in *S. typhimurium* 27 also resembles the multiple resistance found in Japan in the spontaneous loss of resistance from about 0.5% of organisms in a resistant culture.

The resistance reported here differs from that reported from Japan in that there

the most common pattern was resistance to streptomycin, tetracycline, chloramphenicol and sulphonamide. This pattern could segregate into a triple resistance to streptomycin, chloramphenicol and sulphonamide, and single resistance to tetracycline. Triple resistance to streptomycin, tetracycline and sulphonamide is reported to be rare in Japan. In the experiments reported here the only transmissible drug resistance found, other than to streptomycin, tetracycline and sulphonamide all together, was in the tetracycline-resistant strain isolated from patient no. 4 (Table 2).

The Japanese workers (Watanabe & Fukasawa, 1961*b*; Mitsushashi, Harada & Kameda, 1961) found that the multiple resistance of their cultures was eliminated by treatment with acriflavine or acridine orange. On this rested part of their argument that the factor responsible was episomal, for these compounds had already been shown to eliminate another episome, the F factor of *E. coli* (Hirota & Iijima, 1957). In the experiments on resistant *S. typhimurium* 27 reported here, acriflavine was not found to eliminate the drug resistance.

The experiments recorded here show that resistance to the three drugs streptomycin, tetracycline and sulphonamide can be gained by *S. typhimurium* 27 as a result of contact with resistant organisms of another species, and can be spontaneously lost. This strongly suggests that the resistance results from the presence of a transmissible, non-chromosomal factor, or "plasmid" in the sense used by Lederberg (1952). In the absence of evidence to test whether the postulated factor can be attached to the chromosome it cannot be identified as an episome as defined by Jacob and Wollman (1958). (See also footnote in Clark and Adelburg, 1962). Nevertheless it may be a true episome. Triple drug-resistance was found in only four of 141 cultures of enteric bacteria isolated from the same environment as the resistant salmonellae (i.e. the faeces of hospital patients) and for all these the inactive drugs were the same—streptomycin, tetracycline and sulphonamide. These four cultures were not able to transfer their resistance to *Sh. sonnei*, nor were any sensitive organisms found among them, with or without acriflavine treatment. These cultures may quite likely have resulted from multiple mutation, resistance to each drug being selected in turn by antibiotics in the environment. But the identity of their resistance pattern with that of the transmissible resistance suggests that they may be carrying the same genetic factor in a chromosomally integrated form.

The development of multiple drug resistance in enteric bacteria could be very serious from the point of view of clinical medicine and public health, but there is no evidence that in the 1959 epidemic it had any significant effect on the severity of the infection with *S. typhimurium* or on the duration of intestinal carriage.

SUMMARY

Among 309 cultures of *Salmonella typhimurium*, phage-type 27, fifteen, isolated from eight patients, were found to be resistant to the three drugs, streptomycin, tetracycline and sulphathiazole. This triple resistance could be transferred by growth in mixed broth culture to a strain of *Shigella sonnei* and back again to sensitive cultures of *S. typhimurium*. In whole cultures the resistance was stable,

but spontaneous loss could be demonstrated in a small proportion of the organisms in such cultures. No elimination of resistance was demonstrated after treatment with acriflavine. Resemblances to the multiple drug resistance in enteric bacteria reported from Japan are noted.

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REFERENCES

- BOYD, J. S. K. & BIDWELL, D. E. (1957). *J. gen. Microbiol.* **16**, 217.
 CALLOW, B. R. (1959). *J. Hyg., Camb.* **57**, 346.
 CLARK, A. J. & ADELBERG, E. A. (1962). *Ann. Rev. Microbiol.* **16** (in press).
 DATTA, N. & PRIDIE, R. B. (1960). *J. Hyg., Camb.* **58**, 229.
 DAVIS, B. D. & MINGIOLI, E. (1950). *J. Bact.* **60**, 17.
 HARADA, K., SUZUKI, M., KAMEDA, M. & MITSUHASHI, S. (1960). *Jap. J. exp. Med.* **30**, 289.
 HARPER, G. J. & CAWSTON, W. C. (1945). *J. Path. Bact.* **57**, 59.
 HIROTA, Y. & IJIMA, T. (1957). *Nature, Lond.*, **180**, 655.
 JACOB, F. & WOLLMAN, E. L. (1958). *C.R. Acad. Sci., Paris*, **247**, 154.
 LEDERBERG, J. (1952). *Physiol. Rev.* **32**, 403.
 LEDERBERG, J. & LEDERBERG, E. M. (1952). *J. Bact.* **63**, 399.
 MITSUHASHI, S., HARADA, K. & HASHIMOTO, H. (1960). *Jap. J. exp. Med.* **30**, 179.
 MITSUHASHI, S., HARADA, K., KAMEDA, M., SUZUKI, M. & EGAWA, R. (1960). *Jap. J. exp. Med.* **30**, 301.
 MITSUHASHI, S., HARADA, K., HASHIMOTO, H. & EGAWA, R. (1961a). *Jap. J. exp. Med.* **31**, 47.
 MITSUHASHI, S., HARADA, K., HASHIMOTO, H. & EGAWA, R. (1961b). *Jap. J. exp. Med.* **31**, 53.
 MITSUHASHI, S., HARADA, K. & KAMEDA, M. (1961). *Jap. J. exp. Med.* **31**, 119.
 STOCKER, B. A. D. (1960). *Tenth Symp. Soc. Gen. Microbiol.* p. 1.
 WATANABE, T. & FUKASAWA, T. (1961a). *J. Bact.* **81**, 669.
 WATANABE, T. & FUKASAWA, T. (1961b). *J. Bact.* **81**, 679.
 WATANABE, T. & FUKASAWA, T. (1961c). *J. Bact.* **82**, 202.