CHLORAMPHENICOL-RESISTANT STRAINS OF *SALMONELLA TYPHOSA*

I. Induction of Morphological, Cultural, and Antigenic Changes

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Changes that occur in gram-positive organisms coincident to the acquisition of resistance to such antibiotics as penicillin, streptomycin, and aureomycin are well documented (Gezon, 1948; Gezon and Cryst, 1948; Gezon and Fasan, 1950; Ramsey and Padron, 1954). However, with the exception of studies with *Escherichia coli* (Coffey *et al.*, 1950; Bergersen, 1953) the changes in such gram-negative enteric pathogens as *Salmonella typhosa* due to acquired resistance to antibiotic substances have not been critically examined. Those few reports available are beset by many contradictory findings. Voureka (1951) reported morphological and cultural alterations in typhoid organisms obtained from the urine of patients receiving chloramphenicol. Corda (1951), although finding differences in oxidative metabolism between chloramphenicol-sensitive and -resistant strains of *S. typhosa*, claimed that there were no substantial differences in morphology or cultural characteristics. The reports on antigenic differences between resistant and nonresistant organisms are equally contradictory. Levy and Fak (1956) could find no effect on antibody response in sera from nontreated typhoid fever cases as compared with those which had been treated with chloramphenicol. However, Seeliger and Vorlaender (1953) reported that patients receiving chloramphenicol therapy for typhoid fever had altered Widal reactions. During treatment somatic agglutinin titers appeared late or remained absent whereas flagellar titers remained unchanged. It was not known whether this altered response was due to antibody suppression or altered antigenic components in the organisms. Carrere *et al.* (1951) and Burgio and Rosano (1951) reported that antiserum obtained after immunization with drug-fast strains agglutinated resistant bacteria at a higher dilution and *vice versa*. Ponzoni (1954) found that *S. typhosa* resistant to subbacteriostatic levels of chloramphenicol showed reduction in H and Vi antigens, but no change in O antigens. Fahri *et al.* (1956) reported that resistance to chloramphenicol modified the antigenic characteristics of an O hypoagglutinable strain of *S. typhosa* by rendering it O hyperagglutinable and less rich in Vi antigen. These modifications were also obtained using the serum of rabbits immunized with the normal and resistant strains.

With the purpose of clarifying these many divergent reports, the present series deals with a strain of *S. typhosa* which has been made resistant to known amounts of chloramphenicol and a comparison of resistant strains with the parent strain with regard to morphological, cultural, antigenic, metabolic, and virulence characteristics.

MATERIALS AND METHODS

The organism used in this study was isolated from a human carrier and identified as a typically flagellated strain of *S. typhosa* in the VW form. It was found to be susceptible to 0.7 μg of chloramphenicol per ml of medium as determined by the half-maximal growth method (Treffers, 1956). Resistance to chloramphenicol was induced by serial transfers of this organism in brain heart infusion broth (Difco) containing increasing amounts of the antibiotic. Organisms at two levels of resistance were selected for study. The parent susceptible strain will be referred to as the 0 strain, and the resistant strains as the 20 and 200 strains indicating their respective resistance to 20 and 200 μg of chloramphenicol per ml of medium.

The *S. typhosa* somatic antigens were prepared by heating a saline suspension of an 18-hr agar culture at 100 C for 2½ hr. The organisms were then washed, resuspended in 0.5 per cent formolized saline and stored at 4 C. In the preparation of *S. typhosa* flagellar antigens, actively motile cultures in the log phase were inoculated into flasks of brain heart infusion broth. After incubation at 18 hr at 37 C, 0.5 per cent formalin was added and the culture reincubated for an

additional 24 hr. The killed organisms were then washed, resuspended in 0.5 per cent formolized saline and stored at 4 C.

Antisera were prepared by immunizing rabbits with the antigens described above. Rabbits were inoculated in the marginal ear vein on alternate days for a period of 4 weeks. Although individual schedules varied, each animal received graded doses from 0.1 to 1.5 ml. Trial bleedings were made and, when adequate titers were reached as determined by slide agglutination tests, the animals were bled out by cardiac puncture and the sera collected and stored at −12 C until used.

Slide and tube agglutination tests were performed using somatic and flagellar antigens according to the methods outlined by Kaufmann (1950). The use of the rapid slide test was confined to the determination of crude end points. The final titer of an antiserum was obtained by means of the tube method.

Monospecific sera for the somatic antigen analysis of the sensitive and resistant strains were prepared as follows. For monospecific IX antiserum, *S. typhosa* strain 0901 (IX, XII_1, XII_2, XII_3) antiserum was adsorbed first with *Salmonella paratyphosa* A var. *durazzo* (II, XII_1, XII_3) followed by adsorption with *Salmonella reading* (IV, XII_1, XII_2) thus leaving only component IX antibodies in the original serum. The

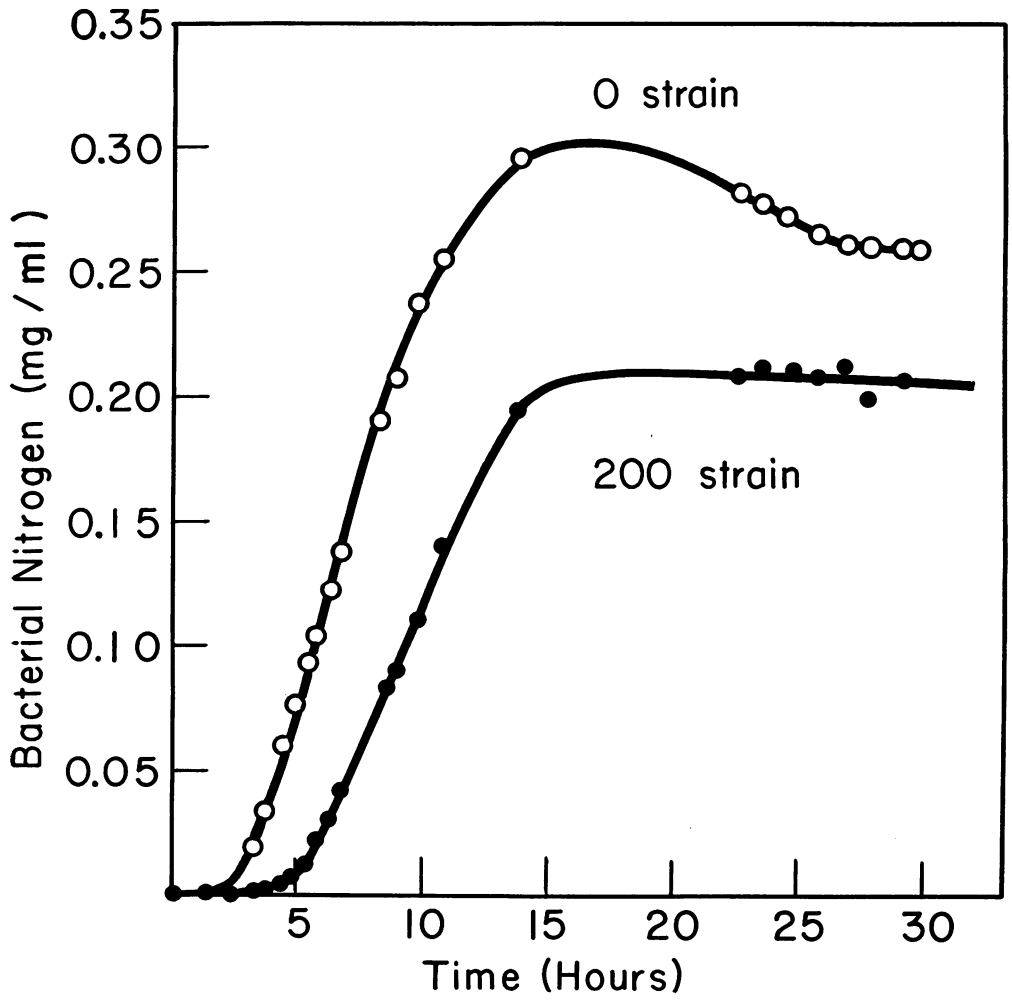

Figure 1. Growth curves of 0 and 200 strains of *Salmonella typhosa* cultured in brain heart infusion broth at 37 C on rotary shaker.

anti-XII_2 serum was prepared by adsorbing *S. typhosa* strain O901 antiserum with *S. typhosa* strain T_2 (IX, XII_1, XII_3) thereby removing all antibodies except those directed against the XII_2 component. The XII_3 serum was prepared by adsorbing *S. paratyphosa* A var. *durazzo* (II, XII_1, XII_3) antiserum with *S. reading* (IV, XII_1) thus leaving antibodies against components II and XII_3, only the latter being able to react with *S. typhosa* antigens.

EXPERIMENTAL RESULTS

Routine cultural and morphological studies revealed only minor differences. As seen in figures 1 and 2, the resistant 200 strain grew at a much slower rate in liquid medium containing chloramphenicol than did the parent strain on antibiotic-free medium. This difference in rate was evident when the organisms were incubated as stationary or shaken cultures. It should be emphasized that the resistant strain need not be cultured in the presence of chloramphenicol to produce the altered growth rate seen above. Essentially identical curves were obtained when the resistant strain was grown in antibiotic-free medium.

When cultured on solid media, the resistant colonies also developed at a slower rate, were somewhat smaller, but otherwise indistinguishable in shape, smooth appearance, and texture from the parent strain.

Examination of Gram stained smears indicated that the resistant strains tended to be more pleomorphic than the parent strain with many bipolar staining rods seen. Electron micrographs confirmed the extreme pleomorphism suggested by the stained preparations. In addition, the shadowed preparations failed to show the presence of flagella on the 200 strain organisms.

To confirm this apparent loss of flagella, 18-hr broth cultures were examined for motility by means of hanging drop preparations. The parent strain was actively motile as is typical for *S. typhosa*. The intermediate resistant 20 strain showed only an occasional motile form, whereas the highly resistant 200 strain was essentially nonmotile. Since it is generally accepted that

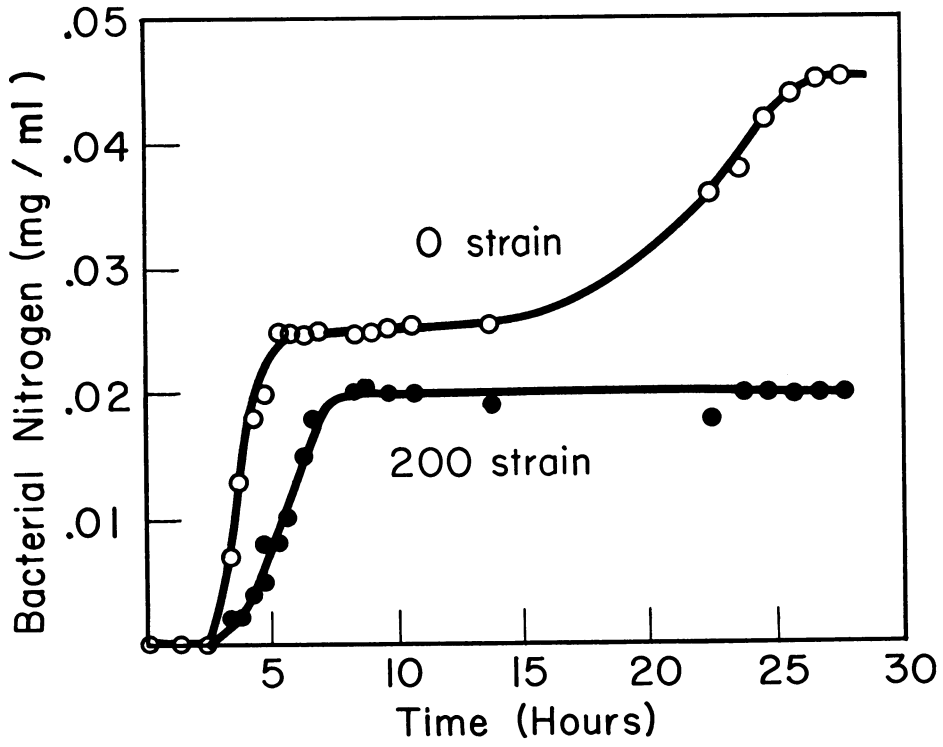

Figure 2. Growth curves of 0 and 200 strains of *Salmonella typhosa* cultured in brain heart infusion broth at 37 C (stationary).

motility is associated with flagella, it was felt that lack of motility would be manifested by a concomitant loss of the flagellar antigen in the resistant organisms. As seen in table 1 when 24-hr cultures were checked against stock Salmonella somatic and flagellar typing sera, the 200 strain had apparently lost its ability to agglutinate in H serum, indicative of loss of the flagellar antigen. The ability of the 20 strain to agglutinate in the H serum was greatly reduced. Since some weakly motile organisms grown at 37 C often appear nonmotile, tests were repeated with cultures grown at room temperature. Under these conditions motility was completely restored to the 20 strain along with the ability to agglutinate in H antiserum. The highly resistant 200 strain became weakly motile at best, and would agglutinate only with extremely high titered flagellar antisera. It may be of interest to note, that this organism remains essentially nonmotile after over 30 transfers in chloramphenicol-free broth.

Experiments were now conducted to determine whether any changes in the somatic antigens had taken place during acquired resistance to chloramphenicol. Rabbits were immunized with suspensions of parent and resistant strains which had been boiled for 2½ hr. Each of the resulting antisera was adsorbed with *Salmonella muechan* (VI, VIII:d, 1, 2) in order to remove antibody directed against the flagellar antigen while leaving all somatic antibodies intact. These somatic antisera were then checked for ability to react with homologous and heterologous antigens by means of both slide and test tube agglutination reactions. The results of these tests are shown in table 2.

The antiserum against the 200 resistant strain was found to react to a titer of 1:10,000 with the homologous antigen but only to 1:200 with the chloramphenicol-susceptible strain. However, when the antiserum against the 0 strain was tested, both antigens were found to react to approximately the same extent. These results indicate at least a quantitative difference between the somatic antigens of the two strains. The possibility exists that during acquired resistance the 200 strain has undergone what Kaufmann (1941) refers to as form variation of the XII somatic antigen with a loss of the XII_2 component. If this variation had occurred, it would in part explain the difference in titers seen with the 200 antiserum, for Kaufmann (1941) has shown that the presence of the XII_2 antigenic component often inhibits agglutination. Inferential evidence can be gained by the examination of results of agglutination reactions using strain O901 which contains antigens XII_1, XII_2, and XII_3. It can be seen that this strain also reacted to a titer 1:200 against the 200 resistant strain antiserum.

To check the possibilities, monospecific antisera directed against somatic antigens XII_2, XII_3, as well as IX, were prepared and the antigenic components of the parent and resistant strains determined by means of the slide agglutination reaction. A crude attempt at quantitation was made by standardizing both antigens turbidimetrically in a Coleman Junior spectrophotometer. As seen in table 3, the parent susceptible strain contains what appears to be the normal complement of antigens IX, XII_2, and XII_3. The 200 resistant strain, however, was found to contain reduced amounts of these antigens in comparison with the parent strain. It

TABLE 1

Agglutination reactions of sensitive and resistant Salmonella typhosa with adsorbed stock antisera

Strain	Somatic Serum*	Flagellar Serum†
0	4+	4+
20	2+	1+
200	2+	0

* Contains agglutinins for somatic antigens IX, XII.

† Contains agglutinins for flagellar antigen phase d.

TABLE 2

Titers of sensitive and resistant anti-Salmonella typhosa sera against homologous and heterologous antigens

Anti-*S. typhosa* Somatic Sera	Test Antigens (Somatic)		
	200	0	O901
200:			
Unadsorbed	10,000*	200	200
Adsorbed with strain 0	2,000	0	0
Adsorbed with O901	1,600	0	0
0:			
Unadsorbed	8,000	10,000	10,000
Adsorbed with strain 200	0	10	10

* Reciprocal of dilution.

TABLE 3

Somatic antigen analysis of sensitive and resistant Salmonella typhosa

Adsorbed Monospecific Antiserum	Titer Using Test Antigen	
	200	0
XII_2	10*	1200
XII_3	100	1000
IX	100	1600

* Reciprocal of dilution.

is possible that the failure of the parent strain to react to the same titer with the 200 strain antiserum is a manifestation of masking due to the presence of a greater amount of the XII_2 antigen. It should be stressed however, that all reactions of the parent strain with these monospecific test sera developed much sooner and to a greater extent than did those with the resistant strain. The possible significance of this finding will be emphasized shortly.

That the difference in somatic antigens may also be a qualitative one can be seen by examining the results of adsorption experiments in table 2. The 200 antiserum was adsorbed once with the 0 strain somatic antigen and checked for remaining agglutinating antibody. Adsorption removed all antibody against both the parent strain and strain O901. However, the antiserum was still able to react to a titer of 1:2000 with the homologous antigen. When the parent strain antiserum was adsorbed with the 200 somatic antigen, practically all ability of this antiserum to react with any of the antigens was lost.

The results of these adsorption studies strongly suggest that during the acquisition of resistance to chloramphenicol a new somatic antigen distinct from antigens IX and XII has evolved in the resistant 200 strain. Attempts to identify and characterize this new antigen are currently in progress.

DISCUSSION

The morphological changes herein described are in keeping with those reported by Coffey *et al.* (1950) in their studies of chloramphenicol-resistant *E. coli*. In addition to extreme pleomorphism, broth cultures of the resistant strain of *E. coli* became nonmotile. Bergersen (1953), using special staining techniques, found that chloramphenicol-resistant *E. coli* contained altered and enlarged nuclear material. In view of the finding of Wisseman *et al.* (1954) that chloramphenicol inhibits protein synthesis, the observation of slower growth rates, the enlarged forms, perhaps representing inhibition of cell division, and the loss of flagella in our resistant strains may represent a partial block in protein synthesis. Further speculation on this point will be withheld until the completion of comparative metabolic studies now in progress.

The qualitative and quantitative changes found to occur in the somatic antigens of the resistant strain are of unusual interest. The qualitative change being the more unusual will be discussed first. The fact that a new antigen was found to develop during the acquisition of resistance to chloramphenicol raises the interesting possibility that this antigen may be a manifestation of the metabolic differences which must exist to enable the organism to grow in an inimical medium. To the best of our knowledge, this is the first report of such a finding in gram-negative microorganisms. It is hoped that the isolation and analyses of this new antigen will shed some light on its possible role in the mechanism of resistance of *S. typhosa* to chloramphenicol.

The quantitative differences as represented by the reduced ability of the parent strain to react with the 200 strain antiserum may in fact be due to higher levels of the XII_2 component as suggested by Kaufmann (1941). That the resistant strain should have undergone at least a partial loss of the XII_2 component during the serial transfers in chloramphenicol is likely since these strains are known to undergo such a form variation spontaneously (Kaufmann, 1941). Another possible explanation for this difference should be considered. The presence of the "new" antigen on the resistant strain may in some way mask the conventional somatic antigens so that an antiserum prepared against it might contain a disproportionately higher level of antibodies directed against the "new" antigen. This hypothesis would also explain the divergency of titers found when the 200 strain antiserum was tested against the parent and resistant strain antigens. It is hoped that current investigations into the nature of the "new" antigen will resolve these problems.

SUMMARY

The acquisition of resistance to chloramphenicol in a strain of *Salmonella typhosa* resulted in

altered morphological and cultural characteristics as well as alterations in somatic antigens. These somatic antigen changes appear to be twofold. One, a quantitative change characterized by a reduction in the amount of somatic antigens XII_2, XII_3, and IX present. And second, a qualitative one characterized by the appearance of a new somatic component not present in the parent strain.

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