A genetical and biochemical study of chlorate-resistant mutants of *Salmonella typhimurium*

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S.typhimurium can form nitrate reductase A, chlorate reductase C, thiosulfate reductase, tetrathionate reductase and formic dehydrogenase. None of these enzymes are formed in chlorate-resistant mutants. Conjugation experiments showed the presence of a strong linkage between the *chl* and *gal* markers of the bacterial chromosome. By deletion mapping the gene order *nic A aro G gal bio chl D uvr B chl A* was found. Strains with deletions terminating between *bio* and *uvr B* or between *uvr B* and *chl A* have a number of aberrant properties. Though resistant against chlorate they reduce nitrate and form gas. After growth with nitrate they form less nitrate reductase than the wild type which may explain the resistance against chlorate. After growth with thiosulfate they form small amounts of thiosulfate reductase and chlorate reductase C. In crosses between an *E.coli* Hfr chl^+ strain and a *S.typhimurium chl A* strain recombinants were obtained, forming nitrate reductase A and chlorate reductase C. These recombinants do not form gas, which indicates that the chl^+ gene from *E.coli* does not function normally in *S.typhimurium*.

INTRODUCTION

Nitrate reductaseless mutants of *Enterobacteriaceae* have recently been isolated in several laboratories. The mutations have been found to be pleiotropic. They cause the loss of nitrate reductase and formate hydrogenlyase in both *Aerobacter aerogenes* (Stouthamer, 1967*a*, *b*) and *Escherichia coli* (Piéchaud et al., 1967). It has been shown that these mutants lack the formate dehydrogenase of the formate hydrogenlyase complex (Stouthamer et al., 1967; O'Hara et al., 1967; Venables, Wimpenny and Cole, 1968). Similar mutants of *Proteus vulgaris* lack nitrate reductase, formate hydrogenlyase, thiosulfate reductase and tetrathionate reductase (Piéchaud et al., 1967).

Chlorate is known to be reduced by nitrate reductase. The products of chlorate reduction are toxic for bacteria. Therefore nitrate reductaseless

mutants are chlorate-resistant. Consequently the genetic symbol *chl* is used for nitrate reductaseless mutants.

Puig and Azoulay (1967) have mapped these mutations. One mutation called *chl A*, was found in the *gal* region of the chromosome of *E. coli*. A second, called *chl B*, was mapped in the *mtl* region. A third locus which does not affect formic hydrogenlyase (most probably the structural gene for nitrate reductase), was observed in the *trp* region. Puig and Azoulay deduced the order *gal-attλ-chl A-bio* from their studies. However, Adhya, Cleary and Campbell (1968) indicate the gene order *gal-bio-chl A*. In addition they found evidence for another *chl* locus (called *chl D*) between *gal* and *attλ*. Venables and Guest (1968) confirmed the results of Adhya et al. (1968) as to the position of the *chl A* and *chl D* markers. Furthermore these authors obtained evidence for the presence of several *chl* genes in the region of the *chl A* marker. Puig et al. (1969) recently confirmed that the *chl A* marker is not located in the *gal-bio* segment.

In this paper it will be described that *Salmonella typhimurium* strains LT2 and LT7 can form nitrate reductase A, chlorate reductase C, thiosulfate reductase, tetrathionate reductase and formate dehydrogenase. All these activities are absent in most chlorate-resistant mutants.

It has been shown that there is a large homology between the chromosomes of *E. coli* and *S. typhimurium* (for review, see Sanderson, 1967). *E. coli* forms only 2 of the enzymes mentioned above for *S. typhimurium*. Therefore it seemed very interesting to compare the *chl*-region of the chromosomes of both organisms and to study the properties of *E. coli–S. typhimurium* hybrids for this region. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Bacterial strains. The properties and origin of the strains used are listed in Table 1. Genetic symbols are those used by Taylor and Trotter (1967) and follow the proposals for a uniform nomenclature in bacterial genetics (Demerec et al., 1966).

Isolation of chlorate-resistant mutants. Mutants resistant against chlorate were obtained by plating bacteria on minimal medium (Hadjipetrou et al., 1964) plates containing ammonia as nitrogen source, cysteine (20 μg/ml) and $KClO_3$ (50 μmoles/ml). Cysteine was added because chlorate interferes with the biosynthesis of this compound in *A. aerogenes* (Stouthamer, 1967*a*). When chlorate-resistant mutants were isolated from auxotrophic mutants the plates were supplemented with the specific growth requirements of the mutant. In all cases plates for the selection of chlorate-resistant mutants were incubated anaerobically in jars filled with 95% N_2 + 5% CO_2. No mutagen was applied

TABLE 1

Characteristics of *S.typhimurium* LT2 and LT7 and *E.coli* strains used

Strain No.	Mating type	Characters	Obtained from
S.typhimurium LT2			
S516	F⁻	*met try*	Dr. H. Mäkelä
S517	Hfr A	*his gal*	Dr. H. Mäkelä
S528, S529, S531	F⁻	*met try chl*	S516
S.typhimurium LT7			
S801	F⁻	*mut fer chlA str*	Dr. C. Colson
S802	F⁻	*mut*⁺ *fer str*	Dr. C. Colson
S808	F⁻	*mut fer chlA str purE*	S801
S812, S813	F⁻	*mut fer chlA str purE gal*	S808
S880	F⁻	*mut*⁺ *fer galE str*	S802
S894	F⁻	*mut*⁺ *fer str del* (*nicA aroG gal bio chlD*)	S880
S896	F⁻	*mut*⁺ *fer str del* (*gal bio chlD uvrB*)	S880
E.coli K12			
H722	Hfr H	*thi purB*	Prof. Dr. P. G. de Haan

as the mutation frequency towards chlorate resistance is unusually high (unpublished results).

Isolation of chlorate-resistant mutants containing deletions. Chlorate-resistant mutants requiring biotin were obtained by plating bacteria on minimal medium as described above but supplemented with biotin (10^{-3} μg/ml). After colonies of chlorate-resistant mutants had appeared the plates were replicated on minimal medium and on minimal medium supplemented with biotin. Auxotrophic mutants resistant against chlorate were obtained by plating bacteria on nutrient broth agar plates containing $KClO_3$ (50 μmoles/ml). After colonies of chlorate-resistant mutants had appeared the plates were replicated on nutrient broth plates and on minimal-medium agar plates.

Isolation of auxotrophic mutants. Auxotrophic mutants were obtained after treatment of the bacteria with N-methyl-N-nitroso-N′-nitroguanidine by the method of Adelberg, Mandel and Chen (1965). After this treatment bacteria were plated on nutrient agar and replicated on minimal-medium agar. Auxotrophic mutants were then isolated and screened for their deficiencies. The *pur* mutation in S808 was identified as *pur E* by the following observations:

1. The mutant produces a brown pigment, which is caused by the accumulation of substituted imidazol derivatives (Stouthamer, de Haan and Nijkamp, 1965).

2. In crosses between *E. coli* Hfr and S808 (see results) many *pur* E^+ recombinants were found to be *lac*$^+$. In *E. coli, pure E* and *lac* are known to be closely linked (Taylor and Trotter, 1967). Such a linkage does not exist for the other mutation causing production of a brown pigment (*pur C*).

Isolation of galactose-negative mutants and of mutants with deletions in the gal region of the chromosome. Galactose-negative mutants were also obtained by plating on minimal-medium plates after mutagen treatment and replication on minimal-medium plates with galactose as sole carbon source instead of glucose as in the normal minimal medium. Some galactose-negative mutants of *S. typhimurium* are known to be extremely sensitive to the presence of galactose (Nikaido, 1961; Fukasawa and Nikaido, 1961). These mutants lack UDP Gal-4-epimerase and accumulate toxic amounts of galactose-1-phosphate and UDP Gal. The galactose-negative mutants were screened for sensitivity to galactose by plating on minimal medium plus glucose (0.2%) and galactose (0.5%) or on nutrient agar plus galactose (0.5%). Of the galactose-negative mutants, S880 was found to be strongly inhibited by galactose. Galactose addition caused lysis as described by Fukasawa and Nikaido (1961) for a UDP Gal-4-epimerase-less mutant. Most probably S880 is blocked in the formation of this enzyme. Nikaido (1961) described that secondary mutations occur eliminating the galactose sensitivity of these mutants. These secondary mutations lead to a loss of galactokinase. On this principle we tried to isolate strains with deletions in the *gal* region of the chromosome. For this purpose S880 (about 10^8 cells per plate) was plated on minimal-salts medium supplemented with glucose (0.2%), galactose (0.5%), nicotinic acid (5 μg/ml), biotin (10^{-3} μg/ml), tyrosine (20 μg/ml), tryptophan (20 μg/ml), phenylalanine (20 μg/ml), *p*-aminobenzoic acid (3 μg/ml), *p*-hydroxybenzoic acid (3 μg/ml) and succinate (20 μmoles/ml). After growth had occurred the plates were replicated on the same medium and on minimal medium with glucose. Most colonies grew on both plates due either to reversion of the original mutation or to deletion of only the *gal* operon. Colonies not growing on the second medium were isolated.

Isolation of mutants containing deletions of the gal and chl region of the chromosome. Mutants of this kind were obtained by plating 10^8 S880 bacteria on nutrient broth containing galactose (0.5%) and $KClO_3$ (50 μmoles/ml). After growth had occurred the plates were replicated and mutant strains were isolated as described above.

Bacteriological tests. Gas formation from glucose and nitrate reduction were determined as described in Cowan and Steel (1965). Thiosulfate reduction was determined by inoculation on slopes of triple-sugar iron medium (TSI) (Cowan and Steel, 1965). These tests correlate with the presence of formic dehydrogenase, nitrate reductase and thiosulfate reductase.

Enzyme determinations. Cell-free extracts were prepared as described before (Stouthamer, 1967*c*). Reductases in cell-free extracts were determined manometrically by a method described by Pichinoty and Piéchaud (1968). In this method H_2 uptake is measured in a system containing: cell-free extract, substrate, benzylviologen and hydrogenase from *Desulfovibrio vulgaris*. Formate dehydrogenase was determined by the method of Peck and Gest (1957) with methylene blue as hydrogen acceptor. Protein was determined as described by Lowry et al. (1951).

Conjugation experiments. Conjugation experiments between *S. typhimurium* donors and acceptors were performed as described by Sanderson and Demerec (1965). Crosses between *E. coli* Hfr strains and *S. typhimurium* F^- strains were performed by a method described by Eisenstark (1965). In this method concentrated, washed suspensions of donor and acceptor bacteria were spread on selective plates. Then a drop of mutagen (saturated solution of N-methyl-N-nitroso-N′-nitroguanidine in 0.067 M phosphate buffer, pH 6.0) was spotted in the middle of the plate. The efficiency of the method is illustrated in Fig. 1.

Transduction experiments. Transduction experiments with phage P22 were performed as described by Blume and Balbinder (1966). In experiments with *chl* recipients direct selection for *chl*$^+$ transductants was made on minimal-medium plates containing sodium lactate (0.25%) as carbon source and KNO_3 (0.35%) as hydrogen acceptor. To improve growth 0.01% casamino acids (Difco) was added. The plates were incubated anaerobically. Only *chl*$^+$ bacteria grow under these conditions.

Genetic analysis of deletion mutants. Determination of galactose and deoxyribose fermentation. The fermentation of these sugars was determined with a medium containing peptone (0.5%), $Na_2HPO_4 \cdot 2H_2O$ (0.434%), KH_2PO_4 (0.066%) and sugar (0.5%). The pH was adjusted to 7.5. After two days of incubation methyl red solution (0.02% in 50% ethanol) was added to detect acid formation.

Nicotinic acid and biotin requirement. The requirement for these vitamins was tested by plating on minimal-medium plates and on minimal medium plates supplemented with these vitamins.

Chlorate-resistance. This was determined by plating on nutrient-broth agar plates with glucose (0.2%) and $KClO_3$ (50 μmoles/ml). The plates were incubated anaerobically.

Determination of UV-sensitivity. A fresh culture of the strain to be tested in nutrient broth was centrifuged. The sediment was resuspended in the same volume buffer suspension, containing 0.067 M phosphate buffer (pH 7.0) and 1 mM $MgSO_4$. The suspensions (3 ml) were irradiated in a petri dish for 60 sec with a germicidal lamp, containing two 15W Philips TL-UV lamps (4 cm apart)

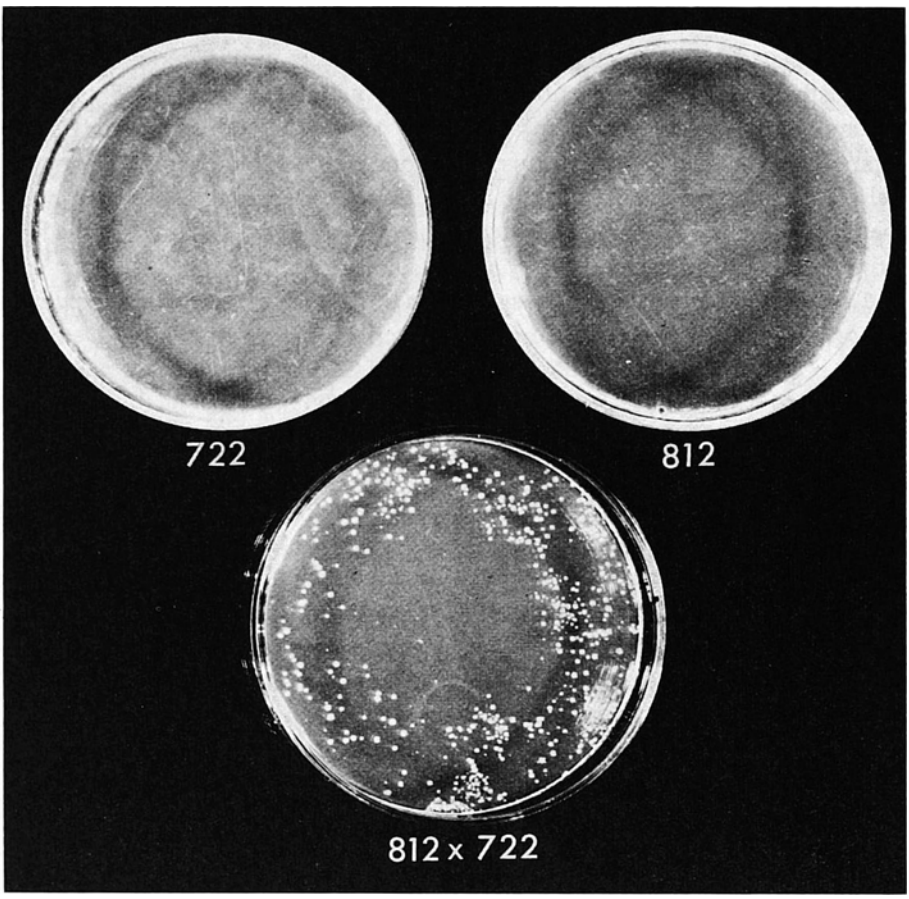

Fig. 1. Efficiency of mutagen-induced mating between *E.coli* Hfr H *thi pur B* (H722) and *S.typhimurium* LT7 F^- *mut fer str pur E gal* (S812). On minimal medium about 10^9 cells were plated of the donor (left), the acceptor (right) and of donor and acceptor (bottom). In the middle of all plates a drop of saturated mutagen solution was placed. The results show the absence of reversion of donor and acceptor and the appearance of recombinant colonies around the drop of mutagen.

at a distance of 70 cm. With uvr^+ bacteria the fraction of surviving bacteria is 4–7%: with uvr^- bacteria it is less than 0.01%.

Determination of the presence of the aro G gene. The *aro G* gene specifies the phenylalanine-repressible 3-deoxy-D-arabinoheptulosonic acid-6-phosphate synthetase (Wallace and Pittard, 1967; Sanderson, 1967). Two other isoenzymes for the same reaction are known. These enzymes are repressed by tyrosine (*aro F*) and by tryptophan (*aro H*) (Wallace and Pittard, 1967). In *aro G* mutants a shortage of phenylalanine will occur when the other two isoenzymes

are repressed. Thus these mutants are sensitive to the presence of both tyrosine and tryptophan. Therefore the presence of the *aro G* gene was determined by plating on minimal medium plus tyrosine and tryptophan.

RESULTS

Reductase formation in S. typhimurium and in E. coli. The specific activities for the reduction of nitrate, chlorate, thiosulfate and tetrathionate by cell-free extracts of *S. typhimurium* and *E. coli* after various growth conditions are shown in Table 2. In *S. typhimurium* the specific activity of nitrate reduction is

TABLE 2

Specific activities of reductases in cell-free extracts of *S.typhimurium* LT2 and LT7 and of *E.coli* strains

Strain No.	Hydrogen acceptor during growth	Specific activity with (μmoles H_2/mg protein per hr)			
		NO_3^-	ClO_3^-	$S_2O_3^{--}$	S_4O^{6--}
S.typhimurium LT2					
S516	None	3.6	8.4	0.7	1.1
	Nitrate	215.7	214.4	0	0
	Thiosulfate	1.6	6.1	1.7	1.1
S528, S529	Thiosulfate	0	0	0	0
S531	None	0.1	0.3	0	not done
	Nitrate	0.2	0.4	0	not done
	Thiosulfate	0.2	0.3	0	not done
S.typhimurium LT7					
S802	None	5.1	12.5	1.2	3.2
	Nitrate	117.7	126.0	0	0
	Thiosulfate	2.9	10.6	3.4	not done
S801	Nitrate or thiosulfate	0	0	0	0
E.coli K12					
H722	None	65.0	58.4	0	0
	Nitrate	194.8	165.6	0	0

strongly increased after anaerobic growth in the presence of nitrate. In *E. coli* this stimulation is much less pronounced. After anaerobic growth in the presence of nitrate both *E. coli* and *S. typhimurium* reduce nitrate and chlorate at about the same rate. Cell-free extracts of *S. typhimurium* after anaerobic growth without hydrogen acceptor or after anaerobic growth with thiosulfate reduce chlorate more rapidly than nitrate. In this case chlorate reduction is catalyzed mainly by a reductase specific for chlorate. A similar enzyme was

detected before by Pichinoty (1965) in a *Hafnia* sp. and was called chlorate reductase C. In cell-free extracts of nitrate-grown cells chlorate reduction is mainly catalyzed by nitrate reductase A. These conclusions are further strengthened by the observation that chlorate reduction in cell-free extracts of nitrate-grown cells is completely inhibited by 1 mM azide, whereas chlorate reduction in cell-free extracts of bacteria grown without hydrogen acceptor is only partially inhibited (Fig. 2). Nitrate reduction is always completely inhibited by

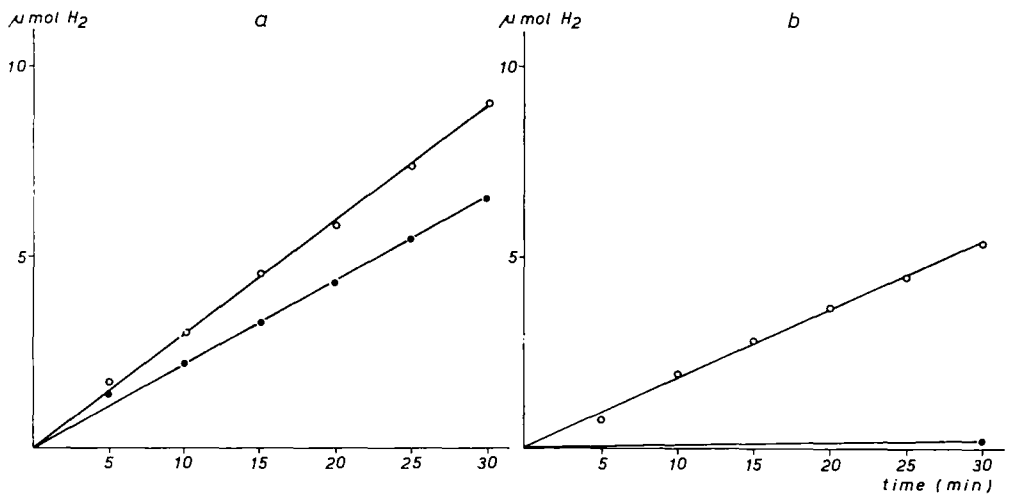

Fig. 2. Influence of 1 mM azide on the rate of chlorate reduction with cell-free extracts of *S.typhimurium*. The bacteria were grown without hydrogen acceptor (*a*) or grown with nitrate (*b*). The Warburg vessels contained 2,2 mg of protein (*a*) and 0.5 mg of protein (*b*) respectively.
o——o : chlorate reduction without azide.
•——• : chlorate reduction in the presence of azide.

1 mM azide. These results show that in cell-free extracts of bacteria grown without hydrogen acceptor chlorate reduction is due to both nitrate reductase A and chlorate reductase C and in extracts of bacteria grown with nitrate only to nitrate reductase A. Extracts of *S. typhimurium* LT2 and LT7 show relatively little thiosulfate-reductase and tetrathionate-reductase activities. In *E. coli* these enzymes are lacking. After growth of *S. typhimurium* in the presence of nitrate these enzymes are not found.

In chlorate-resistant mutants these enzymes are either not formed or the specific activities are extremely small. After growth in the presence of these hydrogen acceptors no increase in the formation of these enzymes was detected (Table 1, S531 and S801). Formate dehydrogenase activity was not detected in

cell-free extracts of chlorate-resistant mutants. In cell-free extracts of S516 grown anaerobically without hydrogen acceptor the specific activity was 4.5 μmoles/mg protein per hr.

Conjugation between chlorate-sensitive and -resistant strains of S. typhimurium. In these experiments S517 acted as donor and S529 as acceptor. In a typical experiment the conjugation mixture was found to contain about 17,000 *met*$^+$ recombinants, 280 *trp*$^+$ recombinants and 40 *met*$^+$ *trp*$^+$ recombinants per ml. This result is in accordance with the point of entry and direction of injection of genetic loci by Hfr A as described by Sanderson and Demerec (1965). *Met* is transferred as an early marker and *trp* as a late marker. The number of *met*$^+$ *trp*$^+$ recombinants is low in comparison with the number of *trp*$^+$ recombinants. This result is in accordance with the observation of several authors that the frequency of crossing-over in *S. typhimurium* is much higher than in *E.coli* (see Sanderson, 1967). *Trp*$^+$ recombinants isolated on minimal medium supplemented with methionine were scored for the presence of the unselected markers *chl*, *gal* and *met*. The result is shown in Table 3. Again the linkage of proximal

TABLE 3

Presence of donor alleles among recombinants of crosses between the *S.typhimurium* strains S517 and S529

Selected marker	Number	Number with donor allele for the marker		
		Chl	*Gal*	*Met*
Trp	265	11	13	26
Trp Gal	13	10		5
Trp Chl	11		10	4

unselected markers is weak. The analysis of the *gal*$^-$ and the *chl*$^+$ recombinants shows that these markers are closely linked. Thus the *chl* marker in *S. typhimurium* is located in the same region of the chromosome as in *E. coli*. Due to the low frequency of inheritance of proximal unselected markers this method is not very suitable for more accurate mapping of the *chl* genes. The linkage of markers is even weaker for the *chl* marker, which may be due to the fact that many *chl* mutants contain small deletions.

Conjugation between an E. coli Hfr chl$^+$ *strain and S. typhimurium LT7 chl mutant.* H722 was used as donor and S801 as acceptor. This strain was chosen as it is known that the mutator gene in *S. typhimurium* increases the frequency of recombinant formation in crosses with *E. coli* Hfr (Miyake, 1962). Strains carrying in addition a second mutation called *fer*, which eliminates restriction of foreign DNA, have even a much higher recipient ability (Colson and Colson,

1967; Okada, Watanabe and Miyake, 1968). It was found that, although the *mut fer* mutations are present in the acceptor strain, the number of recombinants obtained was very low. Even in this case the number of recombinants was greatly increased by the addition of mutagen to the conjugation plates, as described by Eisenstark (1965) for *E. coli* Hfr × *S. typhimurium* LT2 crosses. The results of crosses between H722 and S808, S812 and S813 are shown in Table 4.

TABLE 4

Presence of donor alleles among recombinants of crosses between *E.coli* Hfr *chl*$^+$ and *S.typhimurium* LT7 *chl* mutants

Cross	Selected marker	Number tested	Number with donor allele for the marker	
			Chl	*Gal*
H722 × S808	*Pur E*	192	37	
H722 × S813	*Pur E*	100	27 (all *gal*$^+$)	35
H722 × S813	*Pur E Gal*	100	92	
H722 × S812	*Pur E*	200	2 (all *gal*$^+$)	20
H722 × S812	*Pur E Gal*	100	17	

It is evident that the linkage of unselected markers in these crosses is much higher than in the crosses of Table 3. A high proportion of the *pur E*$^+$ recombinants has inherited the donor *gal* and *chl* markers, although these are transferred later than the *pur E mar*ker by this Hfr. The strong linkage between the *gal* and *chl* marker and the observation that all *chl*$^+$ recombinants from the crosses 2 and 4 are *gal*$^+$ indicate the gene order *pur E – gal – chl*. Experiments with mutants with deletions of this part of the chromosome (see later) show that mutants not forming reductases and formic dehydrogenase are mutated in the *chl A* locus. Consequently S801 bears the *chl A* marker. In the crosses with S808 and S813 the recombinants were stable, indicating that integration of the donor marker in the recipient chromosome had taken place. The crosses between H722 and S812 deviate from the others. The inheritance of the *gal* marker is about normal, but the inheritance of the *chl* marker is very low. Furthermore the *chl*$^+$ recombinants from this cross are unstable and the nitrate-reductase activity in these recombinants is small. Most probably these recombinants are partially diploid. Thus the introduction of the *gal* marker of S812 prevents the integration of the *chl* marker of the donor.

All *pur E*$^+$, *pur E*$^+$ *gal*$^+$ and *pur E*$^+$ *chl*$^+$ recombinants were found to have retained sensitivity to bacteriophage P22. None of the *chl*$^+$ recombinants tested produces gas, which indicates that they do not form formic dehydrogenase. Furthermore they produce no H_2S at all or only traces in TSI medium. Thus

TABLE 5

Specific activities of reductases in cell-free extracts of a recombinant from a cross between H722 and S808

Grown with	Specific activity with (μmoles H_2/mg protein per hr)			
	NO_3^-	ClO_3^-	$S_2O_3^{--}$	$S_4O_6^{--}$
No hydrogen acceptor	4,8	53.6	0	0
Nitrate	75.4	74.3	0	0

they do not form thiosulfate reductase or only very small amounts. The results of enzyme determinations in cell-free extracts of one of these recombinants are shown in Table 5. It is evident that the recombinant forms only chlorate reductase C and nitrate reductase A. With other recombinants similar results were obtained.

Properties of mutants with deletions in the gal region of the chromosome. Nearly all colonies obtained by plating S880 on nutrient broth supplemented with galactose and $KClO_3$ contain deletions of the *gal chl A* region of the chromosome. A number of deletion mutants were obtained by plating S880 on galactose-containing medium. Mutants with deletions of only the *bio* region were obtained by plating S802 on nutrient broth or minimal medium with biotin both supplemented with $KClO_3$. However, only a small fraction (about 0.2%) of the chlorate-resistant mutants appearing on these media turned out to be biotin-requiring. The properties of deletion mutants are shown in Table 6.

TABLE 6

Characteristics of mutants of *S.typhimurium* LT7 with deletions of the *chl* region of the chromosome

Group	Number of strains	Presence of the indicated markers in the deleted region						
		Nic A	*Aro G*	*Gal*	*Bio*	Gas	*Uvr* B	Chl
1	1	–	–	–	–	+	+	r
2	4	–	–	–	–	–	–	r
3	3	+	–	–	+	+	+	s
4	3	+	–	–	–	–	–	r
5	2	+	+	–	–	+	–	r
6	3	+	+	–	–	+	+	s
7	66	+	+	–	–	–	–	r
8	1	+	+	+	–	–	+	r
9	15	+	+	+	–	–	–	r

The signs – and + denote that the indicated marker is deleted respectively is not deleted; r and s denote resistance resp. sensitivity against chlorate.

It is evident that 3 categories of the *chl* mutants obtained are unusual as they produce gas and form nitrite from nitrate (groups 1, 5 and 8). Only traces of H_2S are produced in TSI medium. The specific activities for the reduction of nitrate, chlorate, thiosulfate and tetrathionate in these mutants are given in Table 7. After growth with nitrate these mutants form smaller amounts of

TABLE 7

Specific activities of reductases in cell-free extracts of some aberrant chlorate-resistant mutants of *S.typhimurium* LT7

Strain No.	Hydrogen acceptor present during growth	Specific activity with (μmoles/mg protein per hr)			
		NO_3^-	ClO_3^-	$S_2O_3^{--}$	$S_4O_6^{--}$
S802	None	5.1	12.5	1.2	3.2
(Wild type)	Nitrate	117.7	126.0	0	0
	Thiosulfate	2.9	10.6	3.4	not done
S894	None	3.7	4.9	0.8	1.4
(Group 1)	Nitrate	42.4	56.0	0	0
	Thiosulfate	1.5	0	0	0
S896	None	7.0	8.8	0	1.4
(Group 5)	Nitrate	39.4	43.7	0	0
	Thiosulfate	1.4	2.0	0.5	1.4

nitrate reductase A. This may explain the resistance against chlorate. After growth without hydrogen acceptor the rate of nitrate and chlorate reduction is about the same. This result suggests that these mutants form only small amounts of chlorate reductase C or none at all. After growth with thiosulfate the activities of all reductases are low in comparison with the wild type. In all other cases the correlation between chlorate-resistance and absence of gas, nitrite and hydrogen sulfide production is complete. The properties of the mutant groups 1, 2, 3 and 4 indicate the gene order *nic A aro G gal*. The existence of mutants of group 6 indicates that in *S. typhimurium* no *chl* gene is located between *gal* and *bio* as in *E. coli* (Venables and Guest, 1968; Adhya et al., 1968). The properties of mutants of groups 1, 5 and 8 indicate that a *chl* gene is located between *bio* and *uvr*. Mutants with deletions of the region between the *bio* and *uvr* markers deviate from most of the *chl* mutants. Therefore a second *chl* locus must exist, which is absent in most of the *chl* deletion mutants. In analogy with *E. coli* this locus is called *chl A*. The gene located between *bio* and *uvr B* is called *chl D*. The results lead to the order *nic A aro G gal bio chl D uvr B chl A* (Fig. 3). In none of the mutants isolated the *suc* or *drb* gene were deleted. Most probably essential genes are located between *nic A* and *suc* and between *chl A* and *drb*.

Transduction of chl mutants. Transduction of 22 *chl* mutants was tried with

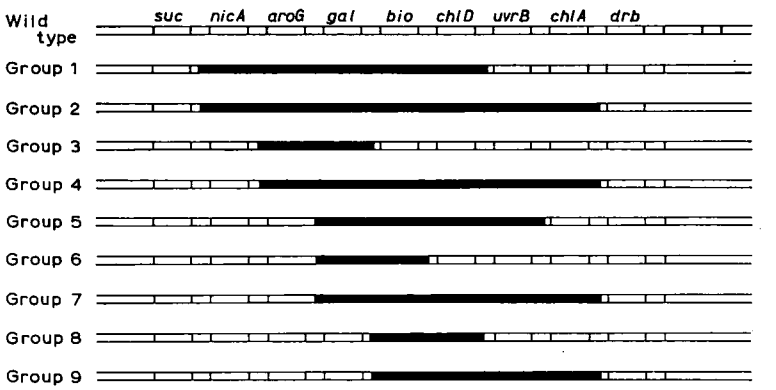

Fig. 3. Gene order in the *suc-drb* segment of the chromosome of *S.typhimurium*. The deleted parts of the chromosome in the various groups of deletion mutants is shown in black. Between the known genes some distance is left open as in many cases other unknown genes will be present between the indicated markers. As a simplification in this figure it has been supposed that in all cases the deletions do not end within a gene.

phage P22 grown in S802. Of these mutants 4 could not be transduced to *chl*$^+$ by this phage. Most probably these mutants contain small deletions preventing transduction. Some of the remaining mutants could not be used in transduction experiments because of their high reversion frequency. These revertants had regained all enzyme activities which were absent in the *chl* mutant. This again indicated the pleiotropic character of these mutations. Of the 22 mutants examined 15 could be transduced by P22 grown in S 802 and also by phage grown in the deletion mutant of group 8. We have also tried 7 deletion mutants of group 9 as donors. Of the transducible strains one was transduced with a high frequency by phage grown in either one of these deletion mutants. Most probably in this mutant a *chl* mutation has occurred in another region of the chromosome. Of the remaining mutants only one (*chl A* 14) was transducible by phage grown on 3 of the 7 deletion mutants of group 9. The explanation of this result is given in Fig. 4. The strains which are not able to

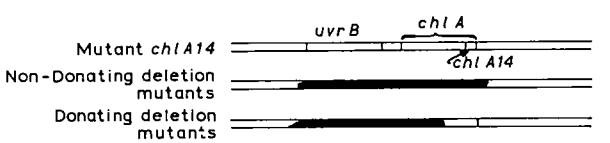

Fig. 4. Transduction of *S.typhimurium chl A* 14 by phage P22 grown in deletion mutants of group 9 (Fig. 3). The *chl A* 14 mutant is placed in the right part of the *chl A* gene. The deletions in mutants able to donate the *chl*$^+$ marker to *chl A* 14 end before this marker. The other deletions not able to donate the *chl*$^+$ marker to *chl A* 14 end to the right of this marker.

transduce *chl A* 14 have deletions which extend further beyond the *uvr B* marker than the deletions of strains which are able to transduce *chl A* 14. This result confirms the existence of a *chl* marker at the place proposed for the *chl A* marker. Of the 15 transducible *chl*-mutants, 13 could not be transduced by phage grown on any of these deletion mutants. This result shows that *chl* mutations mostly occur in the *chl A* region of the chromosome and only rarely in other regions. As these mutants are transduced by phage grown on the deletion mutant of group 8 we may conclude that in this strain the *chl A* region is not deleted.

Properties of mutants with deletions in other regions of the chromosome. Sixteen chlorate-resistant mutants have been isolated which show growth on nutrient-broth agar and not on minimal-medium agar plates. Of these, 6 are able to grow in biotin-supplemented minimal medium. Of the remaining mutants, one requires thiamin and another cysteine. The nutritional requirements of 8 other mutants are very complex and have not yet been determined. These mutants are uvr^+ and consequently in these mutants other regions of the chromosome must be deleted. This result shows again the existence of *chl* markers in other regions of the chromosome.

DISCUSSION

The results presented in this paper show that a high degree of homology exists between the *nic A–chl A* segments of the chromosomes of *E. coli* and *S. typhimurium*. The order *nic A aro G gal* is opposite to the order mentioned for *E. coli* by Taylor and Trotter (1967). However, the order *aro G nic A gal* given for *E. coli* seems to be mistaken (van de Putte, personal communication). For other regions this homology between the chromosomes of these organisms had been found before (Sanderson, 1967). However in *S. typhimurium* no *chl* gene is present in the *gal-bio* segment as in *E. coli*. Instead, a *chl* gene is found between *bio* and *uvr B*. This might be due to an inversion of the *bio-chl D* segment between the two species. Such an inversion has been described before for the *trp-cys B-pyr F* segment of the chromosome (Sanderson and Demerec, 1965; Ino and Demerec, 1968). The *chl D* mutants of *S. typhimurium* form gas and nitrite. Similarly *chl D* mutants of *E. coli* are reported to have some nitrate-reductase activity (Adhya et al., 1968). This agreement gives some support to the inversion hypothesis. More detailed study is necessary however before we may definitely conclude that the *bio-chl D* segment is indeed inverted between the two species.

The number of mutants obtained with deletions terminating between *bio* and *uvr B* and between *uvr B* and *chl A* is very low. Consequently it cannot be inferred that no other *chl* genes occur between *bio* and *uvr B*, nor that other *chl* genes occur at the place of the *chl A* marker.

From these results and the studies of Adhya et al., (1968), Venables and Guest (1968) and Puig and Azoulay (1967) it is evident that many genes are involved in the resistance against chlorate. All these mutations are pleiotropic causing the loss of several enzymes. (Stouthamer, 1967*a*, *b*, Stouthamer et al., 1967; Piéchaud et al., 1967). The hypothesis has been put forward that in these mutants the formation of membranous particles carrying these enzymes is blocked (Stouthamer, 1967*b;* Azoulay, Puig and Pichinoty, 1967). Recent experiments of Azoulay and Puig (1968) and Azoulay, Puig and Couchoud-Beaumont (1969) strongly support this hypthesis. They showed that during incubation of mixtures of high-speed supernatants of cell-free extracts of nitrate-grown *chl A* and *chl B* mutants under special conditions, nitrate-reductase activity appeared. The reconstituted nitrate-reductase activity was found to be located on particles formed during incubation. As many *chl* genes exist we may conclude that many proteins are involved in the formation of these particles.

The high degree of homology between the *E. coli* and *S. typhimurium* chromosomes is also illustrated by the observation that *chl*$^+$ recombinants are obtained upon conjugation between *E. coli chl*$^+$ donors and a *S. typhimurium chl A* acceptor. However, the *chl*$^+$ gene from *E. coli* does not function in *S. typhimurium* as the homologous gene. A similar result has been found for *pyr F*$^+$ recombinants obtained in crosses between *E. coli pyr F*$^+$ donors and *S. typhimurium pyr F* acceptors (Ino and Demerec, 1968). This may be due to the formation of either a hybrid enzyme or a hybrid-enzyme complex unable to function normally.

The way in which these hybrid recombinants are formed is very peculiar. From the results of Miyake (1962) it is known that the *mut* gene is important for obtaining recombinants in these heterologous crosses. The addition of mutagen by the method of Eisenstark (1965) proved to be very successful even though the *mut* gene was present in our acceptor strain. Most probably the *mut* gene and mutagen addition have the same effect in increasing the number of recombinants obtained in these crosses. If mutations are necessary for obtaining recombinants it is understandable that mutagen addition is more efficient. Most probably the effect of mutations on the formation of recombinants in these heterologous crosses is to increase the genetic homology between the genetic regions in question. This conclusion seems to be confirmed by the observation that mutations decreasing this genetic homology (the *gal* mutation in S812) decrease the frequency of recombinant formation. The observation that hybrids between *E. coli* and *Salmonella* exhibit a high recipient ability when used as recipients in crosses with *E. coli* donors points in the same direction (Baron, Carey and Spilman, 1959; Miyake, 1962) This higher recipient ability is due to the presence of integrated *E. coli* genetic material, which

increases the genetic homology between donor and acceptor (Johnson, Falkow and Baron, 1964). More work will be necessary, however, before a complete understanding of all aspects of mutagen-promoted recombinant formation is obtained.

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REFERENCES

ADELBERG, E. A., MANDEL, M. and CHEN, G. C. C. 1965. Optimal conditions for mutagenesis by N′-methyl-N′-nitro-N-nitrosoguanidine in *Escherichia coli* K 12. ← Biochem. Biophys. Res. Comm. **18:** 788-795.

ADHYA, S., CLEARY, P. and CAMPBELL, A. 1968. A deletion analysis of prophage lambda and adjacent genetic regions. ← Proc. Natl. Acad. Sci. U.S. **61:** 956-962.

AZOULAY, E. and PUIG, J. 1968. Reconstitution of enzymatically active particles from inactive soluble elements in *Escherichia coli* K12. ← Biochem. Biophys. Res. Comm. **33:** 1019-1024.

AZOULAY, E., PUIG, J. et COUCHOUD-BEAUMONT, P. 1969. Étude des mutants chlorate-résistants chez *Escherichia coli* K12. I. Reconstitution *in vitro* de l'activité nitrate-réductase particulaire chez *Escherichia coli* K12. ← Biochim. Biophys. Acta **171:** 238-252.

AZOULAY, E., PUIG, J. and PICHINOTY, F. 1967. Alteration of respiratory particles by mutation in *Escherichia coli* K12. ← Biochem. Biophys. Res. Comm. **27:** 270-274.

BARON, L. S., CAREY, W. F. and SPILMAN, W. M. 1959. Genetic recombination between *Escherichia coli* and *Salmonella typhimurium*. ← Proc. Natl. Acad. Sci. U.S. **45:** 976-984.

BLUME, A. J. and BALBINDER, E. 1966. The tryptophan operon of *Salmonella typhimurium*. Fine structure analysis by deletion mapping and abortive transduction. ← Genetics **53:** 577-592.

COLSON, C. and COLSON, A. M. 1967, Host specificity and fertility in *Salmonella typhimurium* LT7. ← Biochem. Biophys. Res. Comm. **29:** 692-695.

COWAN, S. T. and STEEL, K. I. 1965. Manual for the identification of medical bacteria. ← Cambridge University Press, Cambridge.

DEMEREC, M., ADELBERG, E. A., CLARK, A. J. and HARTMAN, P. E. 1966. A proposal for a uniform nomenclature in bacterial genetics. ← Genetics **54:** 61-76.

EISENSTARK, A. 1965. Mutagen-induced hybridization of *Salmonella typhimurium* LT2 × *Escherichia coli* K12 Hfr. ← Proc. Natl. Acad. Sci. U.S. **54:** 117-120.

FUKASAWA, T. and NIKAIDO, H. 1961. Galactose-sensitive mutants of *Salmonella*. II. Bacteriolysis induced by galactose. ← Biochim. Biophys. Acta **48:** 470-483.

HADJIPETROU, L. P., GERRITS, J. P., TEULINGS, F. A. G. and STOUTHAMER, A. H. 1964. Relation between energy production and growth of *Aerobacter aerogenes*. ← J. Gen. Microbiol. **36:** 139-150.

INO, J. and DEMEREC, M. 1968. Enteric hybrids. II. *S.typhimurium–E. coli* hybrids for the *trp-cys B-pyr* F region. ← Genetics **59:** 167-176.

JOHNSON, E. M., FALKOW, S. and BARON, L. S. 1964. Recipient ability of *Salmonella typhosa* in genetic crosses with *Escherichia coli*. ← J. Bacteriol. **87:** 54-60.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. ← J. Biol. Chem. **193:** 265-275.

MIYAKE, T. 1962. Exchange of genetic material between *Salmonella typhimurium* and *Escherichia coli* K12. ← Genetics **47:** 1043-1052.

NIKAIDO, H. 1961. Galactose-sensitive mutants of *Salmonella*. I. Metabolism of galactose. ← Biochim. Biophys. Acta **48:** 460-469.

O'HARA, J., GRAY, C. T., PUIG, J. and PICHINOTY, F. 1967. Defects on formate hydrogenlyase

in nitrate-negative mutants of *Escherichia coli*. ← Biochem. Biophys. Res. Comm. **28**: 951-957.

OKADA, M., WATANABE, T. and MIYAKE, T. 1968. On the nature of the recipient ability of *Salmonella typhimurium* for foreign deoxyribonucleic acids. ← J. Gen. Microbiol. **50**: 241-252.

PECK, H. D., JR. and GEST, H. 1957. Formic dehydrogenase and the hydrogenlyase enzyme complex in *coli-aerogenes* bacteria. ← J. Bacteriol. **73**: 706-721.

PICHINOTY, F. 1965. Recherche des nitrate-réductases bactériennes A et B: méthodes. ← Bull. Soc. Chim. Biol. **47**: 1526-1528.

PICHINOTY, F. et PIÉCHAUD, M. 1968. Recherche des nitrate-réductases bactériennes A et B: méthodes. ← Ann. Inst. Pasteur **114**: 77-98.

PIÉCHAUD, M., PUIG, J., PICHINOTY, F., AZOULAY, E. et LE MINOR, L. 1967. Mutations affectant la nitrate-réductase A et d'autres enzymes bactériennes d'oxydoréduction. Étude préliminaire. ← Ann. Inst. Pasteur **112**: 24-37.

PUIG, J. et AZOULAY, E. 1967. Étude génétique et biochemique des mutants résistant an ClO_3^- (gènes *chl A*, *chl B*, *chl C*). ← C. R. Acad. Sci. **264**: 1916-1918.

PUIG, J., AZOULAY, E., GENDRE, J. et PICHARD, E. 1969. Étude génétique des mutants de la région *chl A* chez l'*Escherichia coli* K 12. ← C. R. Acad. Sci. **268**: 183-184.

SANDERSON, K. E. 1967. Revised linkage map of *Salmonella typhimurium*. ← Bacteriol. Rev. **31**: 354-372.

SANDERSON, K. E. and DEMEREC, M. 1965. The linkage map of *Salmonella typhimurium*. ← Genetics **51**: 897-913.

STOUTHAMER, A. H. 1967*a*. Nitrate reduction in *Aerobacter aerogenes*. I. Isolation and properties of mutant strains blocked in nitrate assimilation and resistant against chlorate. ← Arch. Mikrobiol. **56**: 68-75.

STOUTHAMER, A. H. 1967*b*. Nitrate reduction in *Aerobacter aerogenes*. II. Characterization of mutants blocked in the reduction of nitrate and chlorate. ← Arch. Mikrobiol. **56**: 76-80.

STOUTHAMER, A. H. 1967*c*. Mutant strains of *Aerobacter aerogenes* which require both methionine and lysine for aerobic growth. ← J. Gen. Microbiol. **46**: 389-398.

STOUTHAMER, A. H., BETTENHAUSSEN, C. W., VAN HARTINGSVELDT, J., VAN 'T RIET, J. and PLANTA, R. J. 1967. Nitrate reduction in *Aerobacter aerogenes*. III. Nitrate reduction, chlorate resistance and formate metabolism in mutant strains. ← Arch. Mikrobiol. **58**: 228-247.

STOUTHAMER, A. H., DE HAAN, P. G. and NIJKAMP, H. J. J. 1965. Mapping of purine markers in *Escherichia coli* K12. ← Genet. Res. Camb. **6**: 442-453.

TAYLOR, A. L. and TROTTER, C. D. 1967. Revised linkage map of *Escherichia coli*. ← Bacteriol. Rev. **31**: 332-353.

VENABLES, W. A. and GUEST, J. R. 1968. Transduction of nitrate reductase loci of *Escherichia coli* by phages P1 and λ. ← Mol. Gen. Genetics **103**: 127-140.

VENABLES, W. A., WIMPENNY, J. W. T. and COLE, J. A. 1968. Enzymic properties of a mutant of *Escherichia coli* K_{12} lacking nitrate reductase. ← Arch. Mikrobiol. **63**: 117-121.

WALLACE, B. J. and PITTARD, J. 1967. Genetic and biochemical analysis of the isoenzymes concerned in the first reaction of aromatic biosynthesis in *Escherichia coli*. ← J. Bacteriol. **93**: 237-244.