ネズミチフス菌塩素酸塩抵抗性突然変異株の遺伝分析

誌名	The Japanese journal of genetics
ISSN	0021504X
著者	榎本, 雅敏
巻/号	47巻4号
掲載ページ	p. 227-235
発行年月	1972年8月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

JAPAN. J. GENETICS Vol. 47, No. 4: 227-235 (1972)

GENETIC STUDIES OF CHLORATE-RESISTANT MUTANTS IN *SALMONELLA TYPHIMURIUM*[1]

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Received December 14, 1971

Mutants selected for resistance to chlorate also lose a function of nitrate reduction (Piéchaud *et al.* 1967). This seems to be due to a defect in a redutase system capable of using nitrate as well as chlorate as substrates. The reduction product of chlorate, probably chlorite, is toxic to the wild-type bacteria. In *Escherichia coli*, many chlorate-resistant mutants (*chl*$^-$) and nitrate reductaseless mutants (*nar*$^-$ or *NR*$^-$) have been isolated and the corresponding genetic loci have been mapped between *bio* and *gal* (*chlD*, *narF*), near *bio* (*chlA*, *narD*, *narE*), *trp* (*chlC*, *NR*), and *mtl* (*chlB*) (Puig *et al.* 1967; Puig & Azoulay 1967; Adhya *et al.* 1968; Venables & Guest 1968; Ruiz-Herrera *et al.* 1969). Some *chl* mutations have been found to have a pleiotropic effect on the activity of several oxido-reduction enzymes and have been investigated in relation to complex enzyme systems bound to cell membrane or structural protein components of it (Azoulay *et al.* 1967; Schnaitman 1969). This system seems to be suitable for genetic analysis of membrane components of bacteria. Venables and Guest (1968) devised a lactate-nitrate medium on which the bacteria of wild phenotype can be selected as regards ability to grow anaerobically and made possible genetic analyses of chlorate-resistant and nitrate-reductaseless mutants by transduction and an Hfr cross under anaerobic condition. In the present study chlorate-resistant mutants were isolated from *Salmonella typhimurium* intending primarily to obtain the mutants with the phenotypic characters in addition to chlorate resistance, which are expected from the pleiotropic effect of chlorate-resistant mutation. Most of the *chl* mutants isolated were classified at least into three groups. The two groups of mutant showed good growth on a nutrient or a minimal medium and one of them was cotransducible with *bio* and *gal* genes. The remaining one showed weak growth on the nutrient medium and no growth on the minimal medium. This report describes preliminary genetic mapping of these *chl* loci and characteristic properties of some mutants not described in *chl* mutants of *E. coli*.

MATERIALS AND METHODS

Bacteria and phage

A wild strain, TM2 of *Salmonella typhimurium* and its auxotrophic mutant SJ1926 (F$^-$, *hisF6*, *gal-1001*, *trp-1571*, *bio-71*) were used as parents for *chl* mutants. SW1391 (Hfr, *met*$^-$, *aro*$^-$) is a derivative of *Salmonella abony*, transferring genetic markers to

1) Contribution No. 776 from the National Institute of Genetics, Mishima 411, Japan.

the recipient in the order 0-*leu-gal-bio-trp-his-met* (Mäkelä 1963). Phage P22 (Zinder & Lederberg 1952) was used for transduction. Genetic symbols are as follows: *aro*, aromatic amino acids (in this case phenylalanine and tyrosine); *bio*, biotin; *chl*, chlorate; *gal*, galactose; *his*, histidine; *leu*, leucine; *met*, methionine; *trp*, tryptophan; Hfr, high frequency of recombination donor; F^-, Hfr recipient.

Media

Chlorate agar (CA) medium (Adhya *et al.* 1968) used for mutant selection was nutrient agar (NA) containing 0.2% $KClO_3$ (w/v) and 0.2% glucose (w/v). Chlorate-EMG medium consisting of nutrient EMB (Clowes & Hayes 1968), galactose (1% w/v), $KClO_3$ (0.2%), and agar (1.5%) was used for selection of *gal chl* deletion mutants. Lactate-nitrate (LN) medium (Venables & Guest 1968) used for transduction and mating consisted of M9 salts (Clowes & Hayes 1968) to which KNO_3 (0.4% w/v), 50% sodium lactate (0.7% w/v), vitamin-free casamino acids (0.05% w/v) and agar (1.5%) were added. Nitrate agar consisting of nutrient broth, 0.2% KNO_3 and 1% agar was used for testing nitrate reduction. Nitrite production was tested with sulfanilic acid reagent and α-naphthylamine reagent solutions (*Difco Manual* B106, 1953) on bacteria stabbed into small test tubes containing nitrate agar.

Selection of chl mutants

0.05 ml each of overnight broth cultures starting from single colony isolates of strain TM2 and SJ1926 was plated on a CA plate and incubated for 48 hr at 37°C in a desiccator filled with N_2-gas. Enlarging the colonies by overnight incubation in air, colonies with large and small size were picked and restreaked on a CA medium.

Transduction with phage P22

The mixture of an equal volume of overnight broth culture (approximately 2×10^9 cells/ml) and phage suspension (1×10^{10} PFU/ml) was incubated for 10 min. at 37°C for adsorption and 0.1 ml or 0.15 ml of the mixture was plated on a selective plate. In spot tests, 0.01 ml phage suspension (1–2×10^{10} PFU/ml) was spotted on a sectioned area of the plate previously spread with recipient bacteria. With chl^+ selection the LN plates were incubated for 72 hr at 37°C under anaerobic condition.

Mating condition

Broth cultures grown overnight with gentle aeration were mixed at densities of approximately 2×10^8 cells/ml of donor and 2×10^9/ml of recipient. The mixture was incubated for 2.5 hr at 37°C, diluted 10-fold into minimal medium (M9) and 0.1 ml was plated on each LN plate supplemented with histidine, tryptophan, phenylalanine, tyrosine (final concentration of each, 20 μg/ml) and biotin (0.01 μg/ml). Incubation was carried out for 96 hr at 37°C under anaerobic condition.

Preparation of crude extracts

Cells were harvested from the anaerobic broth culture which contained glucose (0.2%) and KNO_3 (0.1%) and incubated at 37°C in a rubber-stoppered flask bubbling with N_2-gas, and washed twice with buffered saline (0.85% NaCl in 0.01 M phosphate buffer, PH 7.2). The washed cells (approximately 480 mg by wet weight) were suspended in

5 ml of 0.1 M phosphate buffer (pH 7.2), exposed to sonication (20 KC) in ice for 5 min. and centrifuged at 1000×g for 20 min. The supernatant fluid was centrifuged at 20,000×g for 40 min. and its supernatant was stored in the cold as a soluble crude extract. The precipitate was washed once with 0.1 M phosphate buffer, resuspended in 5 ml of the same buffer and stored as an insoluble crude extract.

Enzyme assays

Nitrate reductase was measured by the method of Showe and DeMoss (1968) in which nitrite reduced from nitrate with methylviologen as an electron donor was assayed colorimetrically at 540 mμ. The total protein content of crude extracts was measured by the method of Lowry *et al.* (1951), with bovine albumin powder as standard. One unit of enzyme is defined as the amount reducing 1 mμ mole of nitrate to nitrite in one minute.

RESULTS

Properties of chlorate-resistant mutants

chl mutants were selected from the wild strain, TM2 on the CA medium at a frequency of approximately one per 10^6 cells. Two types of colony, large and small size, were observed. Mutants were so liable to revert that the reversion test was carried out plating about 10^8 cells of each mutant clone on the LN plate and incubating under anaerobic condition. Among 28 mutants with large colony size 11 showed no reversion, 5 few (the order less than 10^{-7} cells) and 12 much reversion (the order of 10^{-6} cells). Most of the revertible mutants indicated pink or red color in the nitrate-reduction tests. The mutants grew well on the minimal medium. Preliminary transduction by spot tests in all pairwise combinations revealed that 16 mutants showing no or few reversions can be divided at least into two groups by the number of chl^+ transductants. Crosses among the mutants of the same group produced no or few transductants, while those between the two groups showed as many transductants as the crosses with the wild strain did. These two classes of mutant were tentatively named as *chlA* and *chlB* because correspondence with the *chl* genes of *E. coli* is unknown. Following mutations belonged to these genes: *chlA* (*1, 2, 3, 5, 9, 12, 13, 14* and *15*) and *chlB* (*4, 6, 7, 8, 10, 11* and *16*). Minute colonies of abortive transductants could not be observed in the cross between the mutants of *chlA* and *chlB* and even in the cross with the wild strain.

Among 25 mutants with small colony size 2 did not revert, 23 showing few reversions. Nitrate reduction tests were negative with all the mutants. These mutants grew weakly on the NA medium and did not grow on the minimal medium. Growth on the latter plate was weakly restored by the addition of NAD (nicotinamide-adenine dinucleotide) or Panvitan (powder of vitamin complex, Takeda) but not by amino acids, bases of nucleic acid, and vitamins (B_1, B_2, B_5, B_6, B_{12}, C, biotin and nicotinic acid). Preliminary transduction in all pairwise combinations among the mutants produced no or few recombinants comparing with transduction from the donors *chlA*, *chlB* and TM2, indicating that these mutation sites may constitute one cluster. The gene was tentatively termed as *chlC* to which the mutants from *chl-17* to *chl-41* belonged.

In the course of growth tests with the colonies selected on the CA medium, the *chl* mutants which show good growth on NA but no growth on the minimal medium were obtained. By auxanography, they were found to require either lysine and methionine (*chl-56, 62*) or cystein and methionine (*chl-57, 59, 60, 61, 63*) for growth on the minimal medium. They were also found to grow weakly on the minimal medium supplemented with NAD (20 μg/ml). The former type of mutant (requiring lysine and methionine) reverted on the minimal medium. The revertants indicated red color in the nitrate-reduction test. In the latter type (requiring cystein and methionine), much revertants appeared on the minimal medium supplemented with cystein or methionine. Some of these revertants indicated red color in the nitrate-reduction test. This may means that the auxotrophic characters of these mutants are coupled with nitrate reduction, that is, the mutation in the *chl* gene has a pleiotropic effect. Detailed characterization of these mutants and their revertants is in progress with genetic analysis. Among the colonies showing no growth on the minimal and the NAD-supplemented minimal medium, chl^- bio^- (*chl-51, 52, 64*) and chl^- bio^- gal^- (*chl-55*) mutants were detected. chl^- gal^- mutants were easily isolated on the chlorate-EMG medium after incubation under anaerobic condition. Almost all of the chl^- gal^- mutants (28 among 32 clones) were bio^- and rough types resistant to phage P22, suggesting that these mutants are deletions covering *chl* and *gal* in which *galE* gene is also involved and results in rough property (Fukasawa & Nikaido 1960). The mutants of *chlA*, *chlB* and *chlC*, and those requiring lysine and methionine for growth on the minimal medium were also isolated from the strain, SJ1926 (*his trp bio gal*) and used for linkage analyses as described below.

Transduction of chl genes

Transduction was quantitatively carried out among representative mutants of *chlA*, *chlB* and *chlC*. *hisE11* and *chl-78*, a derivative of SJ1926 (his^- trp^- bio^- gal^-), requiring lysine and methionine for growth, were added to the experiment. The results are shown in Table 1. Each mutant produced no or few chl^+ transductants in the cross within the group and many transductants in the cross between the groups and with the wild strain. *chl-78* belongs, probably, to the same group as *chlC17*. However, in the reciprocal crosses, $A1 \times A5$, $A3 \times A5$, and $B6 \times B7$, the number of chl^+ transductants is too large as compared with an ordinary recombination frequency of intracistronic transductions which is at most few percent of the control cross with the wild strain. Therefore, the possibility still remains that each tentatively assigned genetic unit is subdivided into cistrons. Lysates of *chlC17* and *chl-78* evoked transductants at higher frequencies in the cross with the mutants of other groups than other lysates including TM2. Titre of lysates prepared from *chlC17* and *chl-78* was always lower by a factor of about 1/5 than that of other lysates when prepared with the same condition, and adjusted to the same titre (1×10^{10}/ml) as other lysates before use for transduction. This phenomenon, therefore, implies that production of transducing particles in these mutant bacteria does not decrease or decreases at a lower rate than plaque-forming particles. In transduction on the LN medium using chl^- bio^- mutants (*chl-51, 52, 64*) as recipients, lysates of $chlA^-$, $chlB^-$ and $chlC^-$ evoked no recombinants. Producing no recombinants even in the transduction from TM2, these chl^- bio^- mutants were inferred

Table 1. Reciprocal transduction among *chl* mutants

Recipient \ Donor	*chlA1*	*chlA3*	*chlA5*	*chlB4*	*chlB6*	*chlB7*	*chlC17*	*chl-78*	TM2
chlA1	0	6 (8.8)	60 (84.9)	532 (764.4)	483 (555.8)	397 (580.4)	3143 (2008.3)	1884	606
chlA3	3 (5.7)	0	75 (106.0)	501 (719.8)	428 (492.5)	359 (524.9)	2485 (1587.8)	1651	545
chlA5	117 (223.7)	94 (137.4)	0	734 (1057.5)	675 (776.8)	559 (817.3)	—	—	533
chlB4	222 (424.5)	250 (365.5)	410 (579.9)	0	0	0	1703 (1088.2)	902	356
chlB6	556 (1063.1)	667 (975.2)	1063 (1503.5)	0	0	53 (77.5)	4564 (2916.3)	2452	753
chlB7	346 (661.5)	443 (647.7)	704 (995.8)	0	41 (47.2)	0	2798 (1787.9)	1611	646
chlC17	202 (386.2)	288 (421.1)	352 (497.9)	266 (382.2)	235 (270.4)	221 (323.1)	0	38	433
chl-78	213 (407.3)	296 (432.8)	330 (466.8)	333 (478.4)	339 (390.1)	259 (378.7)	58 (37.1)	0	636
hisE11	579 52.3%	693 68.4%	716 70.7%	704 69.6%	880 86.9%	693 68.4%	1585 156.5%	494	1013 100%

The number of transductants per 0.3 ml transduction mixture was scored. With *hisE11* the transduction mixture was diluted to 10 times. The number in parentheses was corrected to make the transduction abilities of donors 100, which were calculated as percentages of the number of $hisE^+$ transductants produced by each donor to that produced by TM2. With *chl-78* the correction was not performed because of its *his* mutation.

Table 2. Transduction from TM2 to SJ1993 (*chlA68* his^- trp^- bio^- gal^-)

Selected marker	Number of transductants tested	Number (percent) of transductants with unselected marker(s)			
$chlA^+$	656	bio^-gal^- 443 (67.5)	bio^+gal^- 207 (31.6)	bio^-gal^+ 0(0)	bio^+gal^+ 6 (0.9)
bio^+gal^+	484		chl^+ 14 (2.9)	chl^- 470 (97.1)	

The transduction mixture was plated on the LN medium supplemented with histidine, tryptophan and biotin for chl^+ selection and incubated anaerobically for 72 hr at 37°C. Each chl^+ transductants was examined for unselected markers streaking to the EMB-galactose medium and the minimal medium containing histidine and tryptophan, histidine and biotin or tryptophan and biotin. All the chl^+ transductants were his^- and trp^-. For bio^+ gal^+ selection, the transduction mixture was plated after washing with buffered saline on the minimal medium supplemented with histidine, tryptophan and galactose instead of glucose. Transductants were examined for the *chl* marker on the LN medium supplemented with histidine and tryptophan by anaerobic incubation.

to be deletions with a deleted region being too large for a single phage particle to cover. For testing linkage of the *chl* genes with other markers, transduction was carried out from TM2 to *chlA68*, *chlB65* and *chlC74* which have his^-, trp^-, bio^- and gal^- mutations as well as chl^-. chl^+ transductants selected on the LN medium containing histidine, tryptophan and biotin were examined for the unselected markers. With *chlB65* and

chlC74, no linkage was detected by testing 624 and 674 transductants respectively. With *chlA68*, 31.6% carried bio^+ allele of the donor as well as chl^+, 0.9% carrying bio^+ and gal^+ as shown in Table 2. This indicates that the possible order is either *chlA-bio-gal* or *bio-chlA-gal*. In transduction using bio^+ and gal^+ alleles as selective markers, transductants carrying chl^+ of the donor were only 2.9% and those carrying chl^- were 97.1% (Table 2). This result indicates that the order *chlA-bio-gal* is proper.

Mating with SW1391

For further investigation on linkage, mating with SW1391 (Hfr met^- aro^-) was carried out using SJ1990 (*chlB65* his^- trp^- bio^- gal^-) and SJ1999 (*chlC74* his^- trp^- bio^- gal^-) as recipients. Recombinants seclcted for chl^+ of the donor and met^+ of the recipient were further tested for unselected markers. The results are presented in Table 3. The frequency of recombinations was approximately one per 10^4 donor cells. In the cross between SW1391 and SJ1990 ($chlB^-$), 75% recombinants received bio^+ and gal^+ alleles from the donor as well as $chlB^+$ and 8.8% received only bio^+ and $chlB^+$. The recombinants carrying gal^+ and $chlB^+$ were only 0.2%. Thus, *chlB* is expected to be located near *bio* and *gal*. Among three possible orders, *bio-gal-chlB*, *bio-chlB-gal* and *chlB-bio-gal*, the first order can be excluded by the above data and the second one is also eliminated by the transduction data indicating no linkage between *chlB* and *bio* or *gal*, though *bio* and *gal* are cotransducible. The third order, *chlB-bio-gal*, is only compatible with the above data. The result of the cross between SW1391 and SJ1999 ($chlC^-$) is rather obscure; however, the data shown in Table 3 can be rearranged as follows:

Table 3. Linkage relationship of *chl* genes with unselected markers

SW1391×SJ1990 (*chlB65*)						SW1391×SJ1999 (*chlC74*)					
Unselected makers				No. of recombinants	%	Unselected makers				No. of recombinants	%
his	*trp*	*bio*	*gal*			*his*	*trp*	*bio*	*gal*		
−	−	+	+	450	75.0	+	+	+	−	196	32.7
−	−	+	−	53	8.8	−	+	+	−	147	24.5
−	−	−	−	42	7.0	−	−	−	−	74	12.3
−	+	+	+	19	3.2	−	−	+	−	73	12.2
−	+	+	−	19	3.2	−	+	−	−	28	4.7
+	+	+	−	7	1.2	+	+	+	+	20	3.3
+	+	+	+	6	1.0	+	+	−	−	17	2.8
+	−	+	+	2	0.3	+	−	+	−	17	2.8
−	+	−	+	1	0.2	−	+	+	+	11	1.8
−	−	−	+	1	0.2	+	−	−	−	7	1.2
Total				600	100.1	−	−	+	+	6	1.0
						+	−	+	+	4	0.6
						Total				600	99.9

Donor, SW1391; met^- + + + + chl^+
Recipient, SJ1990 (or SJ1999); + his^- trp^- bio^- gal^- chl^-
Selection was made for chl^+ from the donor and met^+ from the recipient (see text). Unselected markers were examined by the method described in Table 2.

Table 4. Nitrate redutase activity of *chl* mutants

unit	*chlA3*		*chlB4*		*chlC17*		TM2	
	sup	ppt	sup	ppt	sup	ppt	sup	ppt
NO_2 mμ moles/mg protein/min.	0.57	2.68	0.66	2.90	0.96	3.78	149	1045
(%)	(0.38)	(0.25)	(0.45)	(0.28)	(0.65)	(0.36)	(100)	(100)

0.1 ml crude extract was added to 2.4 ml reaction mixture (0.1 M KNO_3, 0.1 mM methylviologen in 0.1 M phosphate buffer, pH 7.2) and preincubated for 5 min. at 37°C. The reaction was started by the addition of 0.1 ml freshly prepared sodium hydrosulfite solution (0.5% in 0.01 M NaOH) and stopped after 10 min. by shaking the mixtur to oxidize hydrosulfite and reduced methylviologen. Nitrite in the mixture was assayed by sulfanilamide and N-1-naphthylethylenediamine reagents colorimetrically at 540 mμ (Showe & DeMoss 1968).

the total frequency of the recombinants receiving at least *his*$^+$ from the donor is 43.4%, the sum of 32.7, 3.3, 2.8, 1.2 and 0.6%. Similarly, those of *trp*$^+$, *bio*$^+$ and *gal*$^+$ are 69.8, 78.9 and 6.7% respectively. *chlC* is expected to be located near the marker showing the high frequency, that is, *trp* or *bio*. Again considering the double markers, the frequencies of *his*$^+$ *trp*$^+$, *trp*$^+$ *bio*$^+$ and *bio*$^+$ *gal*$^+$ are estimated to be 38.8, 67.3 and 6.7% respectively. The inferrence that *chlC* is located between *trp* and *bio* is most plausible. Further, comparing the linkage frequency between *trp*$^+$ and *chlB*$^+$ (8.8%, the sum of 3.2, 3.2, 1.2, 1.0 and 0.2%) with that between *trp*$^+$ and *chlC*$^+$ (69.8%, the sum of 32.7, 24.5, 4.7, 3.3, 2.8 and 1.8%), the order *trp-chlC-chlB-bio* is inferred. In conclusion, considering the data obtained from transduction together, the order of *chl* and other loci involved in this experiment was inferred to be *his-trp-chlC-chlB-chlA-bio-gal*.

Activity of nitrate reductase

Experiments were performed to ascertain the inability of nitrate reduction by *chl* mutants enzymatically and to examine the localization of this enzyme. Supernatant fluids and pellets by high-speed centrifugation at 20,000×g for 40 min. were prepared from *chlA3*, *chlB4*, *chlC17*, and the wild strain, TM2 and used for enzyme assay as the soluble and the insoluble crude extracts respectively. As shown in Table 4, activity of TM2 was 149 units per mg protein in the soluble extract and 1045 units in the insoluble one, and that of the three *chl* mutants was less than 0.7% of the wild strain. The specific activity of the insoluble extract is 7 times higher than the soluble one. The activity of the extracts stored overnight at 4°C decreased to 4.6% in the soluble extract and to 57% in the insoluble one, showing that the enzyme is much more stable in insoluble state.

DISCUSSION

Most of the *chl* mutants examined in this study were classified at least into three groups and the corresponding genes were mapped in the order of *trp-chlC-chlB-chlA-bio-gal* by transduction with phage P22 and mating with the Hfr strain, SW1391. The *chlA* gene was cotransducible with *bio* and *gal*. Abortive transduction could not be

observed in this system. It might be due to technical inadequacy such as a composition of the medium used, because in a *E. coli* system abortive transduction has been reported (Venables & Guest 1968). Therefore, the possibility remains that each of the above *chl* genes is subdivided into more than one cistron by complementation tests. The mutants of *chlA* and *chlB* were indistinguishable phenotypically on the nutrient or the minimal medium from each other, while the *chlC* mutant was different from *chlA* and *chlB* in the respect that it shows weak growth on the nutrient medium and no growth on the minimal medium. Its growth can be restored by supplementing NAD (nicotinamide-adenine dinucleotide) or Panvitan, a commercial vitamin complex. The mutant such as *chlC* has not been reported in *E. coli*. In *E. coli*, so far at least 7 genes concerned with chlorate resistance and nitrate reduction have been reported; *chlA*, *chlB*, *chlC*, *chlD*, *narD*, *narF* and *NR*. It has been reported that *chl* and *nar* mutants are not genetically distinct (Venables & Guest 1968). Among them, both *chlD* and *narF* have been mapped between *bio* and *gal*, and *chlB* has been situated close to *mtl*. These genes were not detected in the present experiments using *S. typhimurium*. However, Alper and Ames (personal communication) and Sanderson (1970) reported the *chl* locus between *bio* and *gal* in *S. typhimurium*. Correspondence between the remaining genes of *E. coli* and the three *chl* genes of *S. typhimurium* is not ascertained.

Nitrate reductase activity in the three types of *chl* mutants was hardly detected. Specific activity of the wild strain was higher and more stable in the insoluble extract than the soluble one, indicating that nitrate reductase is a membrane-bound enzyme as previously reported by Iida and Taniguchi (1959) and Showe and DeMoss (1968).

Enzymatic pleiotropy of the *chl* mutants was not examined in this study; however, the existence of *chlC* mutants and the miscellaneous mutants showing strange properties on the minimal medium indicates the pleiotropy of the *chl* genes. One of the miscellaneous mutants, *chl-78*, required lysine and methionine for good growth and NAD for for weak growth on the minimal medium. Transduction between *chlC* and *chl-78* indicated that *chl-78* is a mutation in *chlC* or a gene closely linked to it. Such the *chl* mutants as express pleiotropy in physiological nature will make it possible to examine genetically the detailed feature of chlorate resistance or nitrate reductase system which is probably bound to structural protein components of the cell membrane. Physiological and genetical studies on these mutants are in progress.

SUMMARY

Chlorate-resistant mutants (*chl*$^{-}$) were isolated from *Salmonella typhimurium* on the nutrient medium containing glucose and $KClO_3$ by the incubation under anaerobic condition. Phenotypic characterization and linkage analyses by transduction with phage P22 and mating with an Hfr strain, SW1391, demonstrated that the *chl* mutants can be classified at least into three groups and the corresponding genetic loci are mapped in the order *trp-chlC-chlB-chlA-bio-gal*, in which only *chlA* is cotransducible with *bio* and *gal*. The *chlC* mutants showed no growth on a minimal medium, which was weakly restored by the addition of NAD (nicotinamide-adenine dinucleotide) or a commercial

vitamin complex. Other pleiotropic mutants requiring lysine and methionine or NAD and those requiring cystein and methionine or NAD for growth on the minimal medium were isolated. Nitrate reductase activity was higher and more stable in the insoluble extract than the soluble one, indicating that this enzyme is membrane-bound.

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