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Hemin-Deficient Mutants of *Salmonella typhimurium*

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Nine hemin-deficient mutants of *Salmonella typhimurium* LT2 were isolated as neomycin-resistant colonies. Five of these mutants could be stimulated by Δ-aminolevulinic acid (Δ-ALA), thus representing *hemA* mutants. Since *S. typhimurium* LT2 is not able to incorporate hemin, the identification of the mutants not stimulated by Δ-ALA was made on the basis of the simultaneous loss of catalase activity and cytochromes. The *hemA* gene was mapped by conjugation in the *trp* region, probably in the order *purB-pyrD-hemA-trp;* the episome FT_{71} *trp* does not carry the *hemA* gene. Transductional intercrosses by phage P22 indicate that *hemA 11*, *12*, *13*, and *37* are at very closely linked sites, whereas *hemA14* is at a more distant site in the same or an adjacent gene. No joint transduction was detected between *hemA* and *trp* or *pyrF*. The loci affected in the other hemin-deficient mutants were linked in conjugation to the pro^+ marker (frequency of linkage, 88 to 97%), but cotransduction of the two markers could not be obtained. The episome F *lac hem purE*, which originates from *Escherichia coli* K-12, could complement these hemin-deficient mutants of *S. typhimurium* LT2. As a result, the sequence of the markers on the chromosome of *S. typhimurium* LT2 is probably *pro heme purE*, analogous to the sequence found in *E. coli* K-12. Thus, the chromosome of *S. typhimurium* also possesses two *hem* regions, with a location similar to that described in *E. coli* K-12.

Research of the last years has shown a great similarity of chromosomal structure between *Salmonella typhimurium* LT2 and *Escherichia coli* K-12, two organisms for which we possess well-elaborated chromosomal maps (3, 11). This is not surprising since the two organisms are closely related and probably originated from a common ancestor, or one species evolved first, with the other having originated from the first by evolution.

Recently we described the position of the *hem* loci on the chromosome of *E. coli* K-12 (9). The hemin-deficient mutants of *E. coli* K-12, selected with neomycin, have provided evidence for the existence of two *hem* regions, one cotransducible with the markers *trp* and *cysB* (locus *hemA*), and the other with the *lac* region (one or more *hem* loci). The probable sequence of markers for the former is *purB*, *hemA*, *trp*, *cysB* (7), and for the latter, *pro*, *lac*, *hem*, *purE* (6).

The comparative position of the *hem* regions in *S. typhimurium* LT2 and in *E. coli* K-12 could not be studied until now, because of the lack of adequate mutants of the former. The aim of this work was to isolate and study genetically the hemin-deficient mutants of *S. typhimurium* LT2. The results of this study, which are given below, show the existence on the chromosome of *S. typhimurium* LT2 of two *hem* regions which correspond in their location to the comparable regions of *E. coli* K-12.

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MATERIALS AND METHODS

Strains. The list of the strains used in this work is given in Table 1.

Nomenclature. The following abbreviations have been used to refer to mutants: *hem*, hemin-deficient; *his*, histidine-requiring; *lac*, lactose-nonutilizing; *met*, methionine-requiring; *ser*, serine-requiring; *str*, streptomycin-resistant; *pro*, proline-requiring; *pur*, purine (adenine)-requiring; *pyr*, pyrimidine (uracil)-requiring; *trp*, tryptophan-requiring; *xyl*, xylose-nonutilizing.

Selection of hemin-deficient mutants. Hemin-deficient mutants were selected with neomycin, which gives a good yield of dwarf-colony mutants in many bacteria (5, 6, 8). Broth cultures of the three strains, LT2 (prototroph), SU18, and SU195 (*proAB47*, *purE*66), grown for a period of 48 to 72 hr at 37 C, were plated on the surface of nutrient agar, containing 50 μg/ml of neomycin. After incubation for 48 to 72

TABLE 1. *Salmonella typhimurium LT2 substrains used*

Strain	Genotype	Sex character	Sequence of transfer of markers	Source
SA536	*serA13* HfrK6	HfrK6	*xyl-ilv-leu-trp*	K. E. Sanderson
SA163	*pyr-210/F$_{13}$ lac purE* (Hirota)	F′		K. E. Sanderson
LT$_2$(prototroph)	Prototroph	F$^-$		L. LeMinor
SU18	*met-365 proA24 pyrD197 purB210 trpE4*	F$^-$		K. E. Sanderson
SU195	*proAB47 purE66*	F$^-$		K. E. Sanderson
LT2	*purE8*	F$^-$		K. E. Sanderson
LT2	*trpD6*	F$^-$		K. E. Sanderson
SA879	*hemA11 met-365 proA24 pyrD197 purB210*	FT$_{78}$	*trp-his-str*	K. E. Sanderson
SU453	*hisF1009 metA22 trpE2 xyl-1 strA201*	F$^-$		K. E. Sanderson
SU688	*pyrF146 cysB12 trpA52 his-1034*	F$^-$		K. E. Sanderson

hr at 37 C, a few barely visible dwarf colonies appeared on the surface of the plates, alongside the resistant colonies of normal size. These dwarf colonies were streaked, and a single-colony was isolated three or four times on nutrient agar without neomycin at 48-hr intervals. The reisolation on media containing hemin cannot be used for the direct identification of hemin-negative mutants, because *S. typhimurium*, like *E. coli* K-12 (9), is not able to incorporate hemin (A. Săsărman, G. Szégli, M. Surdeanu, and A. Dumitrescu, *manuscript in preparation*).

The dwarf-colony mutants, which proved to be stable after several inoculations on nutrient agar without neomycin, were tested qualitatively for the presence of catalase. The catalase-negative mutants were grown in the presence of Δ-aminolevulinic acid, (Δ-ALA), which stimulates the growth of *hemA* mutants. The mutants not stimulated by Δ-ALA were further identified on the basis of absence of catalase activity (quantitative test) and cytochromes. The simultaneous lack of catalase activity and cytochromes in dwarf-colony mutants of *S. typhimurium* selected by neomycin represented the major criterion for the identification of the hemin-deficient mutants not stimulated by Δ-ALA.

Stimulation of growth by Δ-ALA. To verify the stimulation of growth by Δ-ALA, two methods were used. With the first, a drop of a 1% solution of Δ-ALA was streaked across the surface of a nutrient agar plate (the commercial peptone contains only traces of Δ-ALA) and left to soak in for a few minutes; the mutants were then inoculated perpendicularly along the Δ-ALA streak. After incubation of 24 hr at 37 C, the stimulation of growth of *hemA*$^-$ mutants is clearly visible, permitting their identification. The second method consists of the incorporation of Δ-ALA in nutrient agar to a final concentration of 50 μg/ml. This technique was used in particular for the purification of the *hemA* mutants.

Catalase determination. For catalase determination, both a qualitative and a quantitative test were used. For the qualitative test, a drop of a 3% solution of H_2O_2 was applied on the surface of an agar plate of the mutant grown for 48 hr. The quantitative method was as described by Herbert and Pinsent (2).

Determination of cytochromes. The determination of cytochromes was done by the determination of the difference spectra (reduced minus oxidized) of the bacterial suspension by the method described by L. Smith (10). The 48-hr-old agar surface cultures of the mutants were washed twice by saline, and the pellet obtained after centrifugation was diluted to an optical density of 3.5 for the determination of alpha and beta spectra and to 1.7 for the gamma spectrum. The determination of difference spectra was carried out on a Beckman DK-2A double-beam recording spectrophotometer, in the region ranging from 400 to 700 nm with 10 by 10 mm cells.

Mating experiments. Conjugation was carried out by a method described by Sanderson, which consists of mixing cultures in the logarithmic phase in the proportion of 1 ml of donor, 2 ml of recipient, and adding 2 ml of fresh broth. The mixture was centrifuged 30 min at 2,000 × *g* to facilitate the contact of the donor with the recipient, and was then maintained for an hour at 37 C. To select for recombinants, the synthetic medium described by Sanderson and Demerec (4) was used. The recombinants were purified by two successive reisolations on the medium used for selection. Since *S. typhimurium* LT2 cannot incorporate hemin, only Hem$^+$ recombinants were selected, except in the case of *hemA* mutants, which grow normally in the presence of Δ-ALA.

The frequency of recombinants with Hem$^-$ recipient proved to be generally low, preventing the use of interrupted matings for the determination of the time of entry of the markers. Therefore the determination of the location of *hem* markers was based in particular on the results of the analysis of the recombinants. This was made by using the synthetic medium mentioned earlier. The Lac$^+$ character was determined by inoculation of EMB medium containing 0.5% lactose. The donor ability of the Lac$^+$ conjugants, obtained in the mating SA165 × SHSS32 (Table 4), was determined by crossing the conjugants with the strain LT2 (*purE8*) and selecting for purE$^+$ recombinants.

Transduction experiments. The transduction was carried out with phage P22 reproduced on the strain LT2 (*trpD6*). The phage lysate, with a titer of 10^9 to 10^{10} particles per ml, was mixed in equal parts with a logarithmic-phase culture of the recipient, concentrated by centrifugation to about 10^9 cells/ml. The mixture was incubated for 30 min at 37 C to allow for the fixation of the phage, and 0.1 ml of the mixture

was then spread on nutrient agar plates. After an incubation of 48 hr at 37 C, the *hem*$^+$ transductants appeared in the form of normal colonies, easily distinguishable from the feebly growing recipient. The nutrient agar was preferred to a synthetic medium in the selection of *hem*$^+$ transductants because the latter gave a lower frequency of transfer in the preliminary transduction experiments. The purification of transductants was made by two successive reisolations on the synthetic medium used for conjugation. The same medium was used for the analysis of transductants.

hemA experiments. Similar techniques of P22-mediated transduction were used, except for the following. A 0.1-ml amount of phage lysate at a titer of 10^{10} to 5×10^{10} plaque-forming units (PFU) per ml was spread on a plate of minimal medium with 0.1 ml of an overnight culture of the recipient, concentrated by centrifugation to about 10^9 cells/ml.

RESULTS

Isolation of hemin-deficient mutants. Of approximately 1,500 dwarf colonies picked from neomycin agar plates, 300 proved to be dwarf-colony mutants. Among these, about 60 did not revert after several inoculations on nutrient agar plates. The catalase activity determination of these 60 mutants showed the lack of activity only for nine of the mutants. Six of these mutants were stimulated by Δ-ALA (Table 2) and were therefore considered *hemA* mutants. The catalase-negative mutants not stimulated by Δ-ALA were tested again for catalase activity (quantitative test) and for cytochromes. All three mutants proved to be catalase-negative and cytochrome-negative (Table 2) and were considered to be hemin-deficient mutants blocked at some step beyond the synthesis of Δ-ALA.

Locus (or loci) hem (non-hemA). The results of mating experiments with HfrK6 (SA536) in the case of Hem$^-$ mutants not stimulated by Δ-ALA are recorded in Table 3. It is evident that there is a high level of linkage frequency between the marker *hem*$^+$, for which the selection was made, and the nonselected *pro*$^+$ marker (88 to 97%). The remaining Hfr alleles were found in the recombinants with much lower frequency from the marker *purE*$^+$ (18 to 25%) to the marker *trp*$^+$ (0.5%). The marker *met*$^+$, which is located proximally with respect to all other markers in the case of HfrK6 (SA536), was found in only 19% of the recombinants. With respect to the results of the mating experiments with a F′ donor (Table 4), the much lower frequency of transfer of the marker *pro*$^+$ (35 to 63%) should be noted in the mating with the donor HfrK6.

TABLE 2. *Properties of hemin-deficient mutants of Salmonella typhimurium LT2*

Mutant	Genotype	Origin	Catalase activity[a]	Stimulation by Δ-ALA[b]	Cytochromes[a]
SHSM60	*hemA60*	LT2 (prototroph)	−	+	
SHSS11	*hemA11 met-365 proA24 pyrD197 purB210 trpE4*	SU18	−	+	
SHSS12	*hemA12 met-365 proA24 pyrD197 purB210 trpE4*	SU18	−	+	
SHSS13	*hemA13 met-365 proA24 pyrD197 purB210 trpE4*	SU18	−	+	
SHSS14	*hemA14 met-365 proA24 pyrD197 purB210 trpE4*	SU18	−	+	
SHSS21	*hem-21 met-365 proA24 pyrD197 purB210 trpE4*	SU18	−	−	−
SHSS31	*hem-31 proAB$^-$ purE66*	SU195 (*proAB$^-$purE66*)	−	−	−
SHSS32	*hem-32 proAB$^-$ purE66*	SU195 (*proAB$^-$purE66*)	−	−	−
SA776	*hemA37*	LT2	−	+	

[a] Minus iedicates absence of cytochrome or catalase activity.
[b] Plus indicates stimulation of growth by Δ-aminolevulinic acid (Δ-ALA); minus indicates no stimulation.

TABLE 3. *Results of mating experiments with HfrK6 (SA536)*

Donor	Recipient	Selected marker	No. of recipients analyzed	Hfr alleles in recombinants						
				met$^+$	*pro*$^+$	*hem*$^+$	*purE*$^+$	*pyrD*$^+$	*purB*$^+$	*trp*$^+$
				%	%	%	%	%	%	%
SA536	SHSS21	*hem*$^+$	183	19.0	88.6	100		10.3	7.6	0.5
SA536	SHSS31	*hem*$^+$	197		88.8	100	18.7			
SA536	SHSS32	*hem*$^+$	385		97.4	100	25.1			

Also, it should be mentioned that the Hem$^+$ recombinants which received the marker *lac*$^+$ do not unmistakably possess, except in one case, a chromosomal marker.

Testing the donor ability in the 17 Lac$^+$ recombinants obtained in the cross SA163 × SHSS32 (Table 4) showed that all recombinants were able to transfer the marker *purE*$^+$ with a high frequency, thus indicating that the episome F-*lac purE* was present.

SHSS21 (*hem-21*), SHSS31 (*hem-31*), and SHSS32 (*hem-32*) were transduced with P22 phage grown on *trpD6*, and 100 Hem$^+$ transductants were obtained and analyzed for joint transfer of the genes for *met*, *pro*, *purE*, *pyrD*, and *purB*. No joint transfer of these genes was observed, so we may conclude that joint transduction of *hem* with these genes is less than 0.6%.

Mapping the hemA locus. FT_{78}, an F′ factor which mobilizes the chromosome in the order *trp-his-str-leu*, but on which chromosomal genes have not yet been located (Sanderson and Hamplova, *unpublished data*), was transmitted into SHSS11 to produce a fertile Trp$^+$ *hemA11* donor. This donor, SA879 (FT_{78}), was crossed to SU453 (F$^-$) and SU688 (F$^-$), and recombinants were selected and tested for unselected genes, including the *hemA11* allele (Table 5). These data indicate close linkage of *hemA-trp* (48/60 = 80% in SU453, 88/106 = 83% in SU688), and of *hemA-pyrF* (89/106 = 84%), whereas linkage of *hemA-his* was reduced (20/60 = 33% in SU453, 54/106 = 51% in SU688) and linkage of *hemA-xyl* was undetectable (0/58 = 0%). The data were further analyzed for gene order by determining the order which requires the minimum number of quadruple crossover (QCO) events in the merozygote. According to the gene order *hemA-pyrF-cysB-trp-his*, 4 QCO events are required in the SU453 cross (Table 5), and 12 QCO occurrences are required in the SU688 cross. A slightly higher number of QCO events is required to accommodate the data when the gene order is *pyrF-cysB-trp-hemA-his*, i.e., 8 QCO occurrences in the SU453 cross, and 14 QCO occurrences in the SU688 cross. These data suggest, but do not establish, the gene order *hemA-pyrF-cysB-trp-his*.

SA536 (*hem*$^+$) was crossed to SHSS11 (*hemA11*), and *trp*$^+$ recombinants were selected and analyzed for unselected genes. The following linkage was detected: *hemA-trp*, 32/41 = 78%; *hemA-purB*, 10/41 = 24%; *hemA-pyrD*, 20/41 = 49%. Analyses of the QCO frequency indicated the order (*purB-pyrC*) − (*hemA-trp*), with no resolution of the *hemA-trp* order afforded. In a similar cross of SA536 (*hem*$^+$) × SHSS14 (*hemA-14*), the following linkages were detected: *hemA-*

TABLE 5. *Recombinants from crosses of SA879 (FT_{78}) hemA11, selected for His$^+$ and analyzed for unselected genes*

Cross	Class no.	Recombinants[a]						
		hem	*pyrF*	*cysB*	*trp*	*his*[b]	*xyl*	Frequency
SA879 (FT_{78})	1	1			1	1	0	16
hemA11 ×	2	0			0	1	0	32
SU453	3	0			1	1	0	8
	4	1			0	1	0	4
SA879 (FT_{78})	1	1	1	1	1	1		45
hemA11 ×	2	0	1	1	1	1		7
SU688	3	0	1	1	0	1		2
	4	0	0	0	0	1		40
	5	0	0	1	1	1		1
	6	0	0	0	1	1		1
	7	1	1	0	0	1		1
	8	0	1	0	1	1		1
	9	1	0	0	0	1		6
	10	1	1	1	0	1		1
	11	1	0	0	1	1		1

[a] Symbols: 1, donor type for allele; 0, recipient types.
[b] Selected gene from the donor.

TABLE 4. *Results of mating experiments with F_{13}lac (SA163)*

Donor	Recipient	Selected marker	No. of recipients analyzed	F′ alleles in recombinants				
				pro$^+$	*hem*$^+$	*purE*$^+$	*lac*$^+$	Donor ability
				%	%	%	%	%
SA163	SHSS31	*hem*$^+$	205	35.1	100	27.8	17.5	
			36 (*lac*$^+$)	2.7	100	100	100	100
			169 (*lac*$^-$)	42.0	100	12.4	0	0
SA163	SHSS32	*hem*$^+$	192	63.0	100	25.0	8.8	
			17 (*lac*$^+$)	0	100	100	100	100
			175 (*lac*$^-$)	69.1	100	17.7	0	0

trp, 54/67 = 81%; *hemA-purB*, 31/67 = 46%; and *hemA-pyrD*, 31/67 = 46%.

A strain carrying FT_{71}, an F′ factor of *S. typhimurium* derived from HfrB2 and carrying the *trp* genes (Sanderson and Hall, *in press*), was crossed to SHSS11 (Trp⁻ HemA⁻ Met⁻ Pro⁻ PyrD⁻ PurB⁻). Of 39 Trp⁺ recombinants, 25 carried FT_{71} as indicated by high fertility for *trp*⁺, but only one also received the *hemA*⁺ gene; therefore *hemA*⁺ is evidently not carried on FT_{71}.

Transduction studies. To determine whether all of the *hemA* mutants represent closely linked alleles, reciprocal intercrosses were undertaken by P22-mediated transduction. The data in Table 6 indicate that *hemA11*, *12*, *13*, and *37* are all closely linked alleles, whereas *hemA14* gives many recombinants with each of the other alleles. A more detailed test (Table 7) indicates that, when the expected number of transductants from intercrosses is estimated through control transductions, where *hemA11* and *hemA14* are assumed to be nonlinked mutations, the observed number of transductants is 31% of the expected with *hemA11* as the recipient, and 27% with *hemA14* as the recipient. These data suggest that *hemA11* and *hemA14* are mutations in the same transducing fragment. Since an analysis of data of Glanville and Demerec (1) reveals that mutations in the same gene in *S. typhimurium* can give as high as 30% of the wild-type rate of transduction, the *hemA11* and *hemA14* mutants may be in the same gene. Attempts to observe abortive transduction, even in cases in which wild-type phage was used as the donor, were not successful, so complementation analysis was not possible.

Tests for joint transduction between *trp-hemA* and *pyrF-hemA*, undertaken after the observation of linkage by conjugation, gave negative results in all cases. The following numbers of transductants were tested: *hemA11-trpA*, 174; *hemA11-trpC*, 125; *hemA11-pyrF*, 25; *hemA14-trpA*, 155; *hemA14-trpC*, 52; *hemA14-pyrF*, 107.

TABLE 6. *Number of transductants per plate from duplicate plates in intercrosses between hemA mutants*

Recipient strain	No phage	Donor strain					
		hemA11	*hemA12*	*hemA13*	*hemA14*	*hemA37*	*hemA*⁺
hemA11	0	0	0	0	39	0	600
	0	0	0	0	29	0	720
hemA12	0	0	0	0	25		416
	0	0	2	0	26		680
hemA13	0	0	0	0	10	0	440
		1	0	7	9	0	318
hemA14	5	18	45	13	1	0	420
	9	16	60	16	5	1	620
hemA37	0	1	2	0	21	1	520
	2		6	1	15	0	436
cysE396	0	226	224	182	146	32	592
		229	188	158	75	40	520

DISCUSSION

The lack of hemin incorporation by *S. typhimurium* LT2 makes the identification of hemin-negative mutants of this organism difficult, hence explaining the lateness of their description. But in applying the method used for the identification of hemin-deficient mutants of *E. coli* K-12 also unable to incorporate hemin (9), the isolation

TABLE 7. *Number of transductants per plate in intercrosses between hemA mutants*

Recipient strain	No phage	Donor strain		
		hemA11	*hemA14*	*hemA*⁺
hemA11	0	7, 0, 6, 2, 3	184, 192, 94, 262, 162; $\bar{x}$ = 179; expected no.[a] = 583	376, 560, 148, 664; $\bar{x}$ = 432
hemA14	0	828, 952, 936, 1,304, 1,228; $\bar{x}$ = 1,050; expected no. = 3,850	4, 2, 0, 2, 7	1,364, 1,088, 1,640, 1,072, 992; $\bar{x}$ = 1,031
cysE396	0	2,148, 3,400, 2,668; $\bar{x}$ = 2,738	948, 956, 912, 996, 1,128; $\bar{x}$ = 988	664, 1,072, 812, 836, 276; $\bar{x}$ = 732

[a] The expected number is an estimate of the number of transductants expected in the intercrosses when the *hemA11* and *hemA14* alleles are not linked, and is based on corrections using the control transductions. Where *hemA11* is the recipient, 432 × 988/732 = 583. The observed number of transductants can then be compared with the expected number (179/583 = 31%).

of such mutants became possible. In principle, the simultaneous loss of catalase activity and of cytochromes by a mutational event is possible, supposing the loss of the common pyrrolic component, or assuming a nonsense mutation or a mutation in a regulatory gene in which the two activities are controlled by genes in the same operon.

The selection of small-colony mutants of *S. typhimurium* LT2 with neomycin showed that, although the same types of mutants as for *E. coli* K-12 are generally obtained, the distribution of these types is, nevertheless, not similar. Thus, in *E. coli* K-12, the large majority of the small-colony mutants isolated with neomycin are hemin-deficient; for *S. typhimurium*, only a very small proportion of the mutants show the inability to synthesize hemin. In addition, although the proportion of *hemA* among the total number of hemin-deficient mutants of *S. typhimurium* LT2 is high, the isolation of such mutants of *E. coli* K-12 is exceptional. Indeed, until now, only two *hemA* mutants of *E. coli* K-12 are known, both recently described (7, 12). These differences are probably specifically determined since they were found in all the strains tested during repeated experiments.

The possibility of selection of hemin-deficient or quinone-deficient (8) small-colony mutants with neomycin shows the importance of respiratory deficiency for this selection. The respiratory-deficient cells could probably incorporate less drug than the normal cells, thus explaining their survival in conditions where the majority of normal cells are killed.

Mating experiments with HfrK6 located the *hem* mutants not stimulated by Δ-ALA close to *pro*, probably in the sequence *pro hem purE*, with *pro-hem* linkage very high (88.6 to 97.4%). However, no *hem-pro* joint transduction was observed. The *hem* locus carried on the F_{13}*lac* factor of *E. coli* K-12 complements the *hem* mutants of *S. typhimurium*, but this does not reveal the map order, although it does demonstrate that the mutations in the two species are in homologous genes. The gene sequence *pro hem purE*, which corresponds to the order in *E. coli* K-12 of *pro lac hem purE* (6), is the most probable.

The conjugation data from crosses of SA879 (FT_{78}) and HfrK6 (SA536) indicate that *hemA* is closely linked to *trp* and *pyrF*, with the gene order *purB-hemA-trp-his* favored over *purB-trp-hemA-his*. This conclusion is further supported by the fact that FT_{71}*trp*, which also carries part of the region between *trp-his*, does not carry *hemA*. No cotransduction could be detected between *hemA* and *trp* or *pyrF*. Five alleles of the *hemA* gene were tested, four of which (*hemA-11, 12, 13, 37*) were very closely linked, but one (*hemA14*) which was apparently in the same gene but not at the same site. The order of genes in the *hemA* region on the *S. typhimurium* chromosome appears to be the same as in *E. coli* K-12 (7).

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